AD_____

Award Number: DAMD17-97-1-7277

TITLE: Identification of Surrogate Markers for Estrogen Receptor Action in Breast Cancer Cells

PRINCIPAL INVESTIGATOR: Julianne Hall
Dr. David McDonnell

CONTRACTING ORGANIZATION: Duke University Medical Center
Durham, North Carolina 27710

REPORT DATE: July 2000

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2000	Final (1 Jul 97 - 30 Jun 00)

4. TITLE AND SUBTITLE
Identification of Surrogate Markers for Estrogen Receptor Action in Breast Cancer Cells

5. FUNDING NUMBERS
DAMD17-97-1-7277

6. AUTHOR(S)
Julianne Hall
Dr. David McDonnell

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Duke University Medical Center
Durham, North Carolina 27710

E-MAIL:
jmg5@acpub.duke.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

Recent studies have described the identification of novel estrogen and antiestrogen inducible genes with interesting paradigms of regulation. However, due to the paucity of known estrogen receptor (ER) genomic targets, it is still not clear how estrogen displays mitogenic effects in the breast, and how these processes are dysregulated during the onset of hormone resistance. It is clear that the discovery of novel estrogen regulated genes will enable us to better define the key regulators of growth in ER-positive breast cancer cells. In the current study, differential display PCR was used to identify surrogate markers of estrogen and antiestrogen action in human breast cancer cells. A novel transcript was discovered that is upregulated by both the ER agonist 17β-estradiol and the antagonist ICI 182,780. The full length cDNA was cloned and identified as hMIP, the human homologue of the mitochondrial intermediate peptidase (hMIP), an enzyme important in cellular respiration. A second estrogen-responsive cDNA, encoding the control region of the mitochondrial genome, was also discovered. These studies highlight the role of ER pathways in mitochondrial function, and suggest that estrogen-stimulated mitogenesis in breast tissue may involve the enhancement of cellular processes of metabolism and energy generation.

14. SUBJECT TERMS
estrogen receptor (ER), breast cancer, differential display PCR, Estrogen, antiestrogen, hMIP, mitochondrial genome control region

15. NUMBER OF PAGES
76

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction

Breast cancer is the leading cancer among women, and its associated mortality rates have not changed significantly over the past two decades. Evaluation of primary tumors has shown that 70% express the estrogen receptor (ER), which confers estrogen responsiveness to the tissue. It has been well established that estrogen promotes the proliferation of ER-positive breast cancer cells. This action is likely due to the modulation of expression of genes involved in the regulation of growth. However, due to the paucity of known ER regulated genes, it is still not clear how estrogen displays proliferative effects in the breast, and how these processes are dysregulated during the onset of hormone resistance in some patients. Therefore, to elucidate the mechanisms of estrogen action in breast tumors, differential display PCR was used to identify novel ER regulated genes in human breast cancer cells. These studies revealed that estrogen induces the expression of several genes that are involved in cellular metabolism and energy production. Importantly, these findings suggest that estrogen-stimulated proliferation within breast tissues may be mediated by the enhancement of cellular processes of growth by this hormone. In addition, these novel ER regulated genes will serve as surrogate markers of estrogen action in human breast cancer cells.

While it was originally thought that a single estrogen receptor (ERα) was responsible for all of physiological actions of estrogen, the cloning of a second ER (ERβ) indicated that our current models of ER action were incomplete and had to be reevaluated to consider the impact of ERβ. In this regard, it has become clear that in order to understand how cells respond to estrogens and antiestrogens, and how these pathways are

dysregulated during hormonal carcinogenesis, it will be necessary to determine the mechanism of action of each ER subtype and how their activities converge. The recent detection of ERβ in human breast tumors suggested that the mitogenic actions of estrogen could be manifest through both ER subtypes. Therefore, to elucidate the mechanisms of ER action in breast cancer, an evaluation of the molecular pharmacology, transcriptional mechanisms, and cellular actions of the novel ERβ subtype was performed. Using reconstituted ER transcription systems in mammalian cells, it was shown that ERα and ERβ display completely distinct transcriptional responses to estrogens and antiestrogens and that ERβ functions as a transdominant repressor of ERα activity. Furthermore, using the different functional properties of the receptors, a series of ERβ specific peptides that could be used to block ERα and/or ERβ transcriptional activity. Overall, these studies have defined components of the complex signaling pathways of the two human ER subtypes and have provided surrogate markers of estrogen action in human breast cancer cells.

Body

Part 1: Identification of Novel Estrogen and Antiestrogen Regulated Genes from Human Breast Cancer Cells

Introduction:

Breast cancer is the leading cancer among women, and its associated mortality rates have not changed significantly over the past two decades (Love and Koroltchouk, 1993). Evaluation of primary tumors has shown that 60% are ER-positive (Wittliff, 1984), in contrast with normal mammary epithelial cells where ER is poorly expressed. While the role of the receptor is not clear, estrogen displays mitogenic effects in ER-positive breast cancer cells. This action is likely due to the modulation of expression of key regulators of growth, such as transforming growth factor-β (TGF-β), a direct target of ER. For the past two decades, antiestrogens such as tamoxifen have been used to block the ER-mediated induction of growth factors and consequently, oppose the proliferative effects of estrogen and halt disease progression in breast cancer patients (Jordan and Morrow, 1999). However, while tamoxifen is a first-line therapy for the treatment of hormone-dependent breast cancer, most women develop resistance to treatment within five years (Marshall, 1995). Many models of tamoxifen resistance have been proposed, which include estrogen receptor mutations, changes in the levels of paracrine growth factors, and the modulation of downstream events in ER pathways (Morrow and Jordan, 1993). However, it has become clear that we need to first define the intracellular targets of estrogen and tamoxifen action in order to understand how cell growth processes escape from endocrine control during antiestrogen therapy.

Two forms of the human estrogen receptor have been described, the widely characterized ERα, and the more recently identified ERβ (Kuiper *et al.*, 1996; Mosselman *et al.*, 1996). Interestingly, both receptors are expressed in breast tumors (Dotzlaw *et al.*, 1996; Leygue *et al.*, 1998); however, ERα is the more predominant subtype, and ERβ is more prevalent in normal mammary tissue (Leygue *et al.*, 1998). These initial observations led to the hypothesis that the mitogenic effects of estrogen in the breast are manifested through ERα. However, this theory has been challenged by recent observations that tumors coexpressing the two receptors are node positive and of a higher grade (Spiers *et al.*, 1999b). Additional studies revealed that ERβ is upregulated in tamoxifen resistant tumors (Spiers *et al.*, 1999a) and that there is an inverse relationship between ERβ and PR (a marker of response to endocrine therapies) expression in breast tumors (Dotzlaw *et al.*, 1999). Cumulatively, these studies suggest that ERβ may be a poor prognostic factor in breast cancer and that it is possible that both ERα and ERβ play a role in estrogen-mediated carcinogenesis.

While several ER targets have been previously described, the new advances in gene hunting technology have facilitated the identification of additional genes with interesting paradigms of ER regulation. Specifically, it was found that the MCP-1 (monocyte chemoattractant protein 1) mRNA is downregulated by estrogen through a mechanism in which an agonist-bound ER antagonizes NF-κB binding to the MCP-1 promoter (Greene, 1997). Others have reported that ER enhances expression of the complement 3 gene product through the classical ERE-mediated pathway in response to estrogen and tamoxifen (Norris *et al.*, 1996). However, since most of the known estrogen and antiestrogen regulated genes have not been shown to be direct ER targets, it is

apparent that there are upstream events in the ER pathway that need to be explored. It is believed that the identification of additional estrogen and antiestrogen regulated genes will (1) advance the general understanding of ER biology, (2) enable us to determine the roles of ERα and ERβ in hormonal carcinogenesis in the breast, and (3) permit a study of how these pathways are dysregulated in tamoxifen-resistant breast tumors.

Materials and Methods:

Biochemicals

DNA restriction and modification enzymes were obtained from Boehringer Mannheim (Indianapolis, IN), New England Biolabs (Beverly, MA), or Promega Corp. (Madison, WI). PCR reagents were obtained from Perkin-Elmer (Norwalk, CT) or Promega Corp. 17β-estradiol, 4-hydroxytamoxifen, and cycloheximide were purchased from Sigma Chemical Co. (St. Louis, MO). The estrogen receptor antagonist ICI 182,780 was a gift from Dr. Alan Wakeling (Zeneca Pharmaceuticals, Macclesfield, United Kingdom). Hybond-N+ nucleic acid transfer membranes, ^{32}PdCTP, and ^{33}PdCTP were purchased from Amersham (Arlington Hts, IL). The random prime labeling kit was purchased from Boehringer Mannheim. The multiple tissue northern blot was purchased from Clontech (Palo Alto, CA).

RNA isolation and northern blot analysis

Total cellular RNA was harvested from MCF-7 cells and T47D cells using the Ultraspec RNA reagent according to the manufacturer's protocol (Biotecx Laboratories, Inc., Houston, TX). mRNA was isolated from total RNA using the PolyATtract mRNA isolation system (Promega Corp.) Twenty μg of total RNA or 2 μg mRNA was separated by denaturing gel electrophoresis, transferred to nitrocellulose membranes, and hybridized with random-prime-labeled cDNA probes for 24 h in 10 ml of hybridization solution (50% formamide, 5x Denhardt's, 1% SDS, 5x SSC).

Differential display PCR

Differential display PCR (Liang and Pardee, 1992) was performed using the GenHunter (Nashville, TN) RNAimage system according to the manufacturer's

protocols. Briefly, MCF-7 cells were treated with 50 μM cycloheximide, and 10^{-7}M 17β-estradiol, 4-hydroxytamoxifen, or ICI 182,780 for 6 h, after which cells were harvested for total RNA. Each RNA sample was purified, DNAse treated, and ethanol precipitated. The RNA was reverse transcribed to cDNA in three reactions, each containing a different one base anchored poly T primer at the 3' end ($AAGCT_{11}G$, $AAGCT_{11}A$, or $AAGCT_{11}C$). Subsets of cDNAs were amplified using 30 random upstream 13bp primers in combination with each of the 3 poly T primers, and each PCR reaction was duplicated. The amplified cDNAs for each hormone treatment group (17β-estradiol, 4-hydroxytamoxifen, and ICI 182,780) were run adjacently (including duplicates) on 6% polyacrylamide sequencing gels. ^{33}PdCTP was used in the PCR reactions to permit the autoradiographic detection of cDNAs. Differences in the expression of individual genes were observed visually and quantitated with a phosphoimager. All differentially displayed bands were excised from the gels, and the cDNAs were extracted as follows: gel slices were incubated for 10 min in 100 μl dH_20 at 22^0C, boiled for 15 min, and spun for two minutes in a table top centrifuge. The supernatant was ethanol precipitated and used for PCR reamplification with the original primers. Prior to further analysis, the differential display PCR was repeated using the same primers that yielded each of the target cDNAs to verify that their hormone regulation was authentic. Those cDNAs that fit these criteria were reamplified, cloned into the TA vector (Invitrogen, Carlsbad, CA), and sequenced using the T7 sequenase version 2.0 sequencing kit (USB Corp., Cleveland, OH). The hormonal regulation of each cDNA was verified by northern blotting.

Plasmids

PGEM3-36B4 (containing the cDNA encoding the 36B4 ribosomal RNA) was a gift from Pierre Chambon. PBSII-mGAPDH (containing the mouse glycerophosphate dehydrogenase cDNA) was created as follows: the PBSII plasmid (Stratagene, Kingsport, TN) and the mGAPDH cDNA (obtained from RT PCR) were digested with EcoRI and ligated together.

Cell culture

MCF-7 and T47D cells were maintained in dulbecco's minimal essential medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum (Life Technologies, Inc.).

Results:

Identification of estrogen and antiestrogen regulated cDNAs from human breast cancer cells

The mitogenic effects of estrogen in breast tissue are thought to mediated through the upregulation of specific genes involved in cellular processes of proliferation. These effects are receptor-dependent, as ER-negative breast tumors do not respond to hormone. In this study, differential display PCR (ddPCR) (Liang and Pardee, 1992) was used to identify novel estrogen and tamoxifen regulated genes in human breast cancer cells. Specifically, MCF-7 cultured cells were used, because this human breast cancer derived cell line demonstrates growth and gene expression when administered estrogen in a capacity that mimics hormonal effects on ER-positive tumors *in vivo* (Gottardis *et al.*, 1988b). Furthermore, MCF-7 cell derived tumors have been used to study hormone resistance in animal models (Gottardis *et al.*, 1989; Gottardis *et al.*, 1988a), indicating that these models could be used in the future to define those genes that escape endocrine control when cells acquire resistance.

For the differential display analysis, MCF-7 cells were administered estrogen or tamoxifen for six hours, after which they were harvested for total cellular RNA. It was shown that a six hour treatment time was sufficient for the induction of ER target genes in pilot experiments where MCF-7 cells were administered the ER agonist 17β-estradiol for different lengths of time. Under these conditions, six hours was sufficient for the induction of two ER regulated genes examined, PS-2 and the human progesterone receptor (data not shown). The concern with using longer hormone treatment was that alterations in gene expression would occur as a secondary effect of cell proliferation.

This phenomenon was apparent in initial experiments where after 24 hours of hormone stimulation, several candidate ER regulated cDNAs were identified which were subsequently found to be upregulated as a secondary effect of downstream pathways. Cell cycle analysis of MCF-7 cells treated with estrogen for various time points revealed that a six hour hormone induction was not sufficient to induce cell proliferation (data not shown). Another objective of the study was to identify direct targets of ER action. This was addressed by coadministering hormone with cycloheximide at a concentration known to inhibit greater than 95% of all cellular protein synthesis (Brown *et al.*, 1984).

One caveat in the use of MCF-7 cells is that a significant fraction of their ER population displays ligand-independent activity, resulting in the expression of estrogen regulated genes in the absence of hormone. This phenomenon has been attributed to activation of ER by signaling pathways induced by dopamine, growth factors and cAMP (Smith, 1998). However, since the success of ddPCR relies on the ability to distinguish between the patterns of gene expression among different hormone treatment groups, the technique requires low basal levels of ER activity for comparative purposes between RNA populations from untreated and hormone treated cells. However, this problem was avoided in the current study by using cells that had been administered ICI 182,780 as the "no hormone" group. This compound is a pure antagonist that prevents ER activation and transcription of its target genes. Furthermore, ICI 182,780 decreases the basal levels of ER activity by inhibiting both ligand-independent and dependent receptor activity (Willson *et al.*, 1997).

Using these modifications, ddPCR was used to compare RNA populations from MCF-7 cells treated with 17β-estradiol, 4-hydroxytamoxifen, and ICI 182,780 (Fig. 1).

Initially, 24 candidate estrogen and/or tamoxifen regulated cDNAs were identified. However, when each was used to probe northern blots of RNA from MCF-7 cells treated with different ER ligands, only two cDNAs reproducibly displayed hormone regulation. The expression patterns of these cDNAs, ERRT-16 and ERRT-24 (ER-regulated transcripts 16 and 24), are shown in Figure 2. ERRT-16 appeared to be either downregulated by estradiol, or upregulated by the antiestrogens ICI 182,780 or 4-hydroxytamoxifen. In contrast, the ERRT-24 message was induced upon estradiol administration. These ER regulated transcripts were brought forward for further analysis.

Figure 1. Differential display PCR technology.

The differential display technique was developed for the identification of differences in gene expression between distinct RNA populations (Liang and Pardee, 1992). Its use permits the comparison of gene transcription between unique cell types, developmental stages or as in the current study, cells that have been administered different drug or hormone treatments. For ddPCR analysis, total cellular RNA is reverse transcribed to cDNA, and the partial cDNA sequences are amplified using different sets of primers. This ensures that only a set of 50-100 transcripts is amplified at once, which permits the display of individual cDNAs on sequencing gels. ^{33}PdCTP is used in the PCR reactions to allow for the autoradiographic detection of cDNAs. The cDNAs for each group to be compared are run in adjacent lanes, and differences in the expression levels of specific genes are visually identified. In this study, ddPCR was used to simultaneously compare the gene expression patterns of MCF-7 cells induced with either ICI 182,780 (ICI), 17β-estradiol (E2), or 4-hydroxytamoxifen (4-OHT).

Figure 1

Differential Display PCR

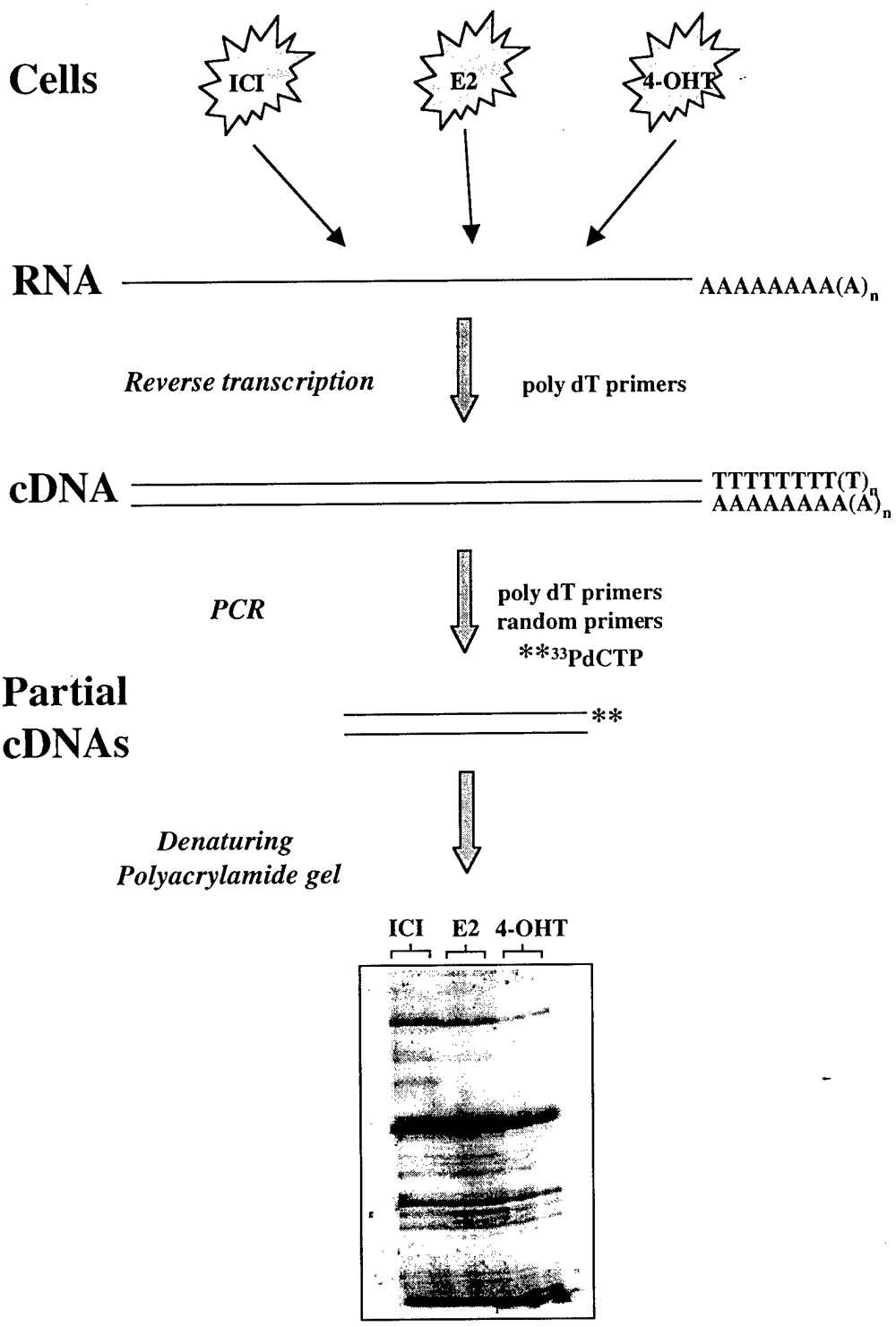
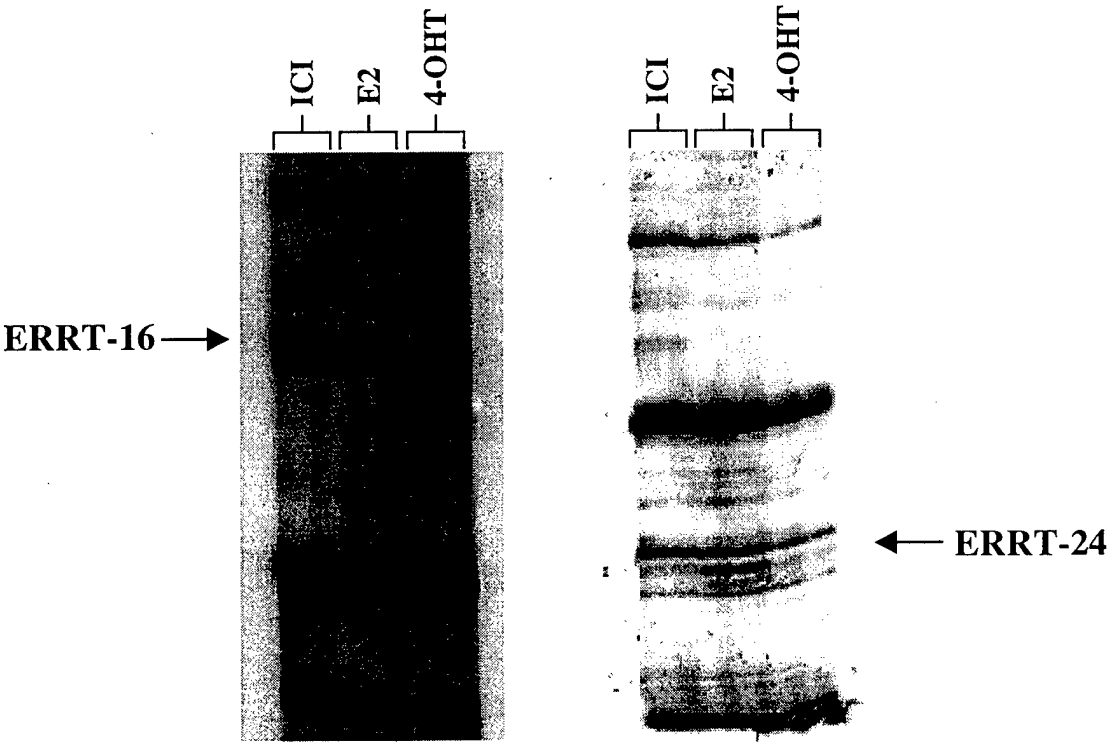


Figure 2. Identification of estrogen and antiestrogen regulated cDNAs from human breast cancer cells.

MCF-7 cells were induced with either 10^{-7} M 17β-estradiol (E2), 4-hydroxytamoxifen (4-OHT), or ICI 182,780 (ICI) in the presence of 50μM cycloheximide for six hours. Total cellular RNA was isolated and used for ddPCR analysis as described. Shown here are two ddPCR gels containing the ER regulated transcripts ERRT-16 and ERRT-24 (ER-regulated transcripts 16 and 24). ERRT-16 expression is decreased in the RNA population from 17β-estradiol treated MCF-7 cells compared to that observed from antiestrogen treated cells. In contrast, ERRT-24 cDNA is upregulated by estradiol.

Figure 2



Characterization of ERRT-16 and cloning of the full length cDNA

The hormone regulation of ERRT-16 was first characterized. The 230bp ERRT-16 transcript obtained from ddPCR was used as a probe of total cellular RNA derived from MCF-7 and T47D human breast cancer cells treated with vehicle, ICI 182,780, or 17β-estradiol for six hours. While the ddPCR results suggested that ERRT-16 was either downregulated by estradiol or upregulated by antiestrogens, northern blot analysis revealed that the transcript was induced both by estradiol and ICI182,780, although to a greater extent in the presence of the pure ER antagonist (Fig. 3A and B). Interestingly, hormonal regulation of ERRT-16 was not observed in Ishikawa (human endometrial carcinoma) and HeLa (human cervical carcinoma) cells (data not shown), two ER-negative cell lines, suggesting that the ER protein was required for the regulation of this transcript by estrogen and antiestrogen.

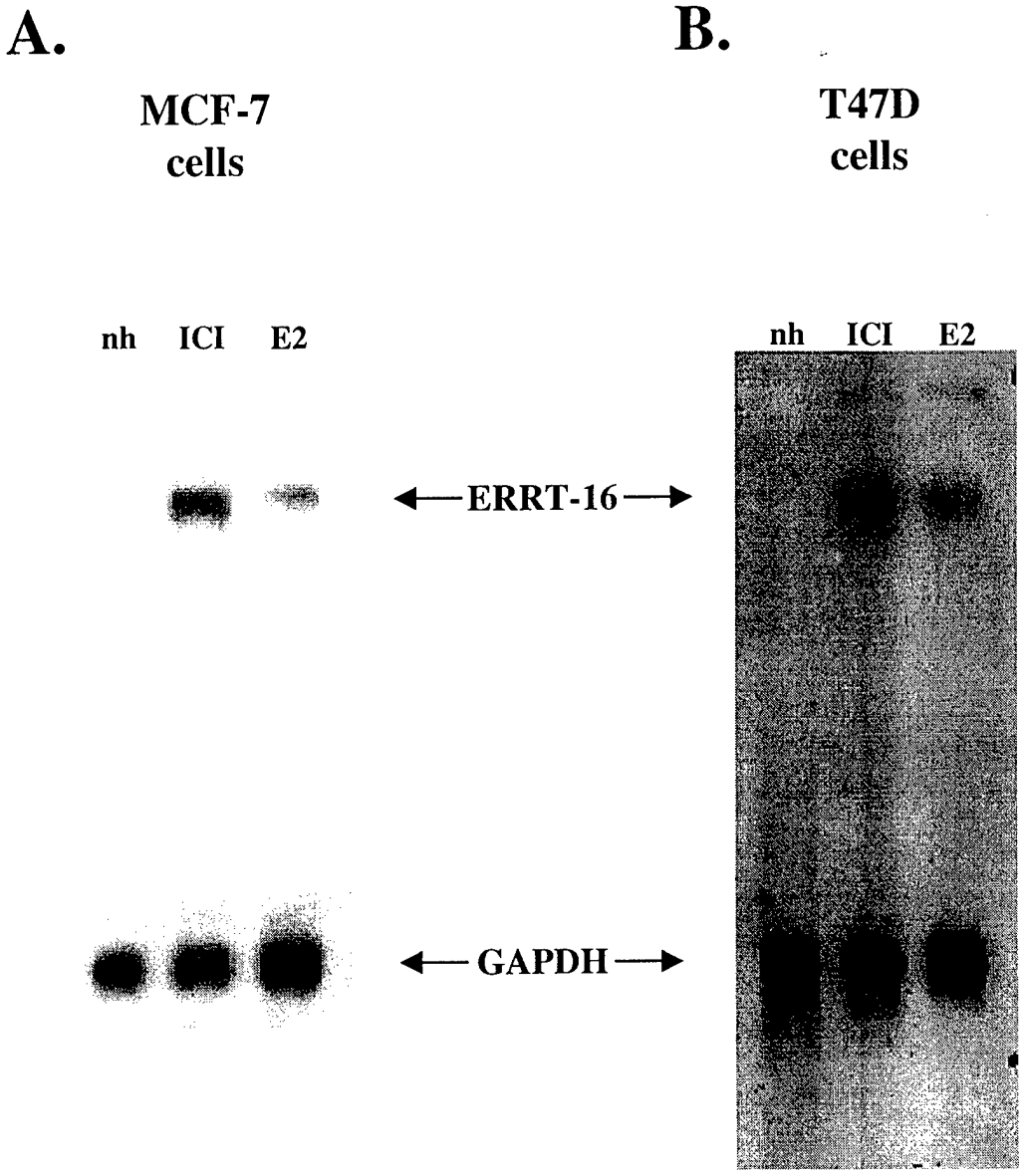
One of the most surprising observations was that the ERRT-16 probe detected a very high molecular weight message (greater than 9 kb) on northern blots of total RNA from MCF-7 and T47D cells, and other human cell lines (Fig. 3; data not shown). Sequence analysis revealed that ERRT-16 corresponded to a previously unidentified transcript. To clone the full length cDNA, ERRT-16 was used to screen an MCF-7 cell cDNA library. Several overlapping clones were identified, together providing approximately 2.4 kb of sequence. RACE PCR was used to map the 5' and 3' ends of the cDNA, which verified that the full length transcript was 2392 bp. However, these findings were puzzling in view of the observation that ERRT-16 hybridized to a very high molecular weight message in the RNA populations of several human cell lines. When the 2392 transcript was used as a probe in northern blots of total RNA, the high

molecular weight message was once again detected, while no visible signal was observed within the 2.4 kb region (data not shown). These observations raised the possibility that ERRT-16 was detecting a more abundant alternatively spliced or unspliced transcript in these experiments. Therefore, to increase the sensitivity of the system and to eliminate unprocessed messages from the RNA pool, the ERRT-16 partial cDNA and the 2392 bp full length transcript were used to probe northern blots of MCF-7 cell mRNA. Under these conditions, a 2.4 kb message was detected by either probe, and the high molecular weight signal was absent (data not shown). These results suggested that the large transcript corresponded to an unspliced form of ERRT-16. In support of this possibility, upon further analysis of the ERRT-16 230 bp ddPCR product, a perfect consensus splice site was identified. Interestingly, this site divided the ERRT-16 transcript into two regions- 50 bp which was identical to a 50 bp sequence contained within the 3' end of the 2.4 kb full-length cDNA, and approximately 180 bp which displayed no homology to any region within the 2.4 kb transcript. Furthermore, this 180 bp sequence was not contained within any the clones obtained from cDNA library screening, even though it comprised most of the probe sequence. In view of these observations, it is likely that the 180 bp sequence corresponds to intronic DNA, and that the 230 bp ERRT-16 transcript was derived from an unprocessed mRNA precursor.

Figure 3. Characterization of the hormonal regulation of ERRT-16.

MCF-7 (**A**) and T47D cells (**B**) were treated for six hours with 50μM cycloheximide and either vehicle (nh), 10^{-7} M ICI 182,780 (ICI), or 10^{-7} M 17β-estradiol (E2), and total RNA was collected. Equal amounts of total RNA (20 μg) for treatment each group was separated on denaturing gels and transferred to a nitrocellulose membrane. The MCF-7 cell RNA blot was probed with the random-prime-labeled ERRT-16 partial cDNA and then stripped and reprobed with the labeled mouse glycerophosphate dehydrogenase (GAPDH) cDNA, used as an RNA loading control. The T47D cell RNA blot was simultaneously probed with the random-prime-labeled ERRT-16 partial cDNA and the GAPDH control. The ERRT-16 probe hybridizes to a high molecular weight RNA species (>9 kb).

Figure 3



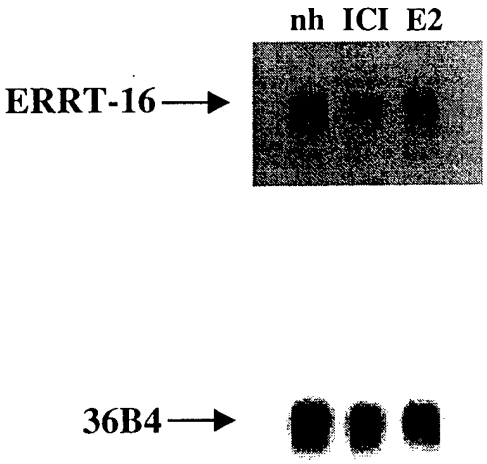
ERRT-16 displays a unique paradigm of regulation

In view of the observation that the high molecular weight ERRT-16 transcript was upregulated by estrogen and ICI182,780 in these studies, the next objective was determine whether the processed transcript (2.4 kb) displayed a similar pattern of regulation. To address this question, the full length ERRT-16 (2392 bp) was labeled used to probe a northern blot of mRNA from MCF-7 cells treated with vehicle, 17β-estradiol or ICI 182,780 for six hours. Surprisingly, under these conditions, a 2.4 kb message was detected which displayed no apparent estrogen- or antiestrogen-mediated induction (Fig. 4). Similar results were seen using a probe synthesized from the ERRT-16 partial cDNA that was obtained from the ddPCR (data not shown). Therefore, after six hours of hormone treatment it appears that ER regulation of ERRT-16 is limited to the unprocessed transcript, suggesting that the receptor may be involved in regulating the stability or splicing of this gene. It is still unclear what the physiological relevance of these findings is, however, it is possible that the expression of the 2.4 kb message is also enhanced by ER pathways under conditions which were not examined (please see Conclusions).

Figure 4. The processed ERRT-16 transcript does not display ER regulation after six hours of hormone treatment.

MCF-7 cells were treated for six hours with 50μM cycloheximide and either vehicle (nh), 10^{-7} M ICI 182,780 (ICI), or 10^{-7} M 17β-estradiol (E2), and total RNA was collected. mRNA was isolated from total RNA, and equal amounts of mRNA (2 μg) from each treatment group was separated on denaturing gels and transferred to a nitrocellulose membrane. The full length ERRT-16 cDNA (2392 bp) was random-prime-labeled and used to probe the mRNA blot. The 36B4 ribosomal protein (36B4) cDNA probe was used as an RNA loading control.

Figure 4



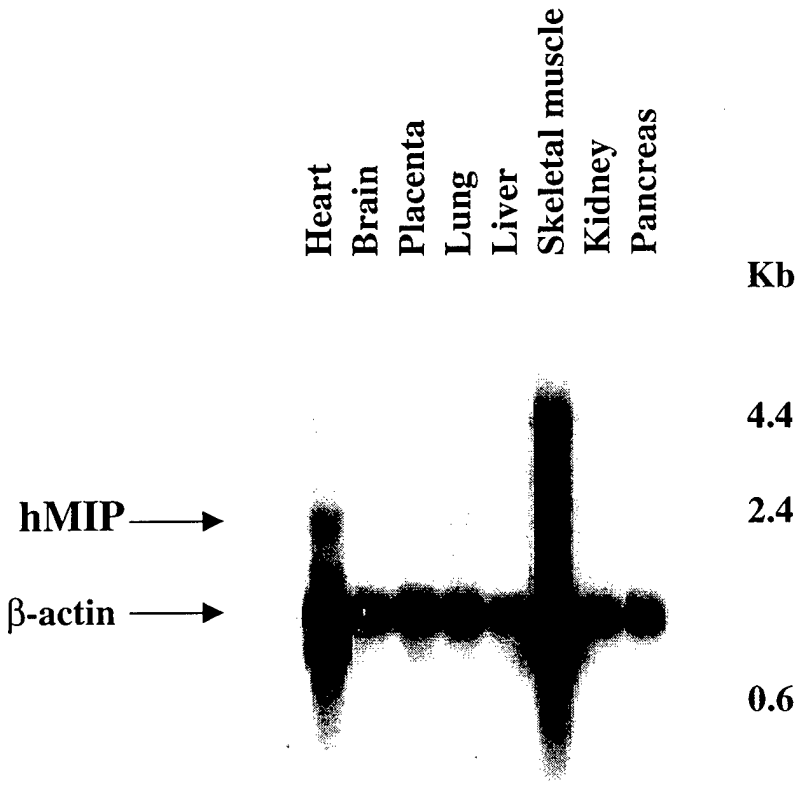
ERRT-16 (hMIP) is primarily expressed in metabolically active tissues

Although the identity of ERRT-16 was not known during the time that cDNA library screening was initiated, Chew *et al.*, (1997) have since reported the identification of a novel 2.4 kb transcript which was identical to the 2392 bp clone identified in this study. Specifically, they discovered that this transcript encodes the human homologue of mitochondrial intermediate peptidase (hMIP). MIP is an enzyme that processes and activates mitochondrial enzymes involved in cellular respiration and mitochondrial replication. These include oxidative enzymes, components of the electron transport chain, the mitochondrial genetic machinery, and mitochondrial translation factors (Branda and Isaya, 1995). Therefore, as predicted, MIP expression correlates with an increase in the processes involved in cellular energy generation and growth. In this regard, it is possible that ER induction of hMIP in human breast cancer cells is one of the mechanisms by which estrogen displays its mitogenic effects in this tissue. To determine whether there was a correlation between hMIP levels and the relative degree of metabolic activity displayed by a particular cell type, expression of hMIP was examined in several human tissues using a Clontech multiple tissue mRNA northern blot (Fig. 5). Strikingly, hMIP was most abundant in heart and skeletal muscle, while it was barely detectable in other tissues examined. These results suggest that hMIP is primarily expressed in very metabolically active tissues. Therefore, the ability of estrogen to enhance the expression of this gene in human breast cancer cells fits with what is known about the proliferative effects of the hormone in this tissue. However, the significance of hMIP upregulation by the ER antagonist ICI 182,780 is not yet clear (please see Conclusions).

Figure 5. ERRT-16 (hMIP) is primarily expressed in metabolically active tissues.

The ERRT-16 (hMIP) cDNA was random-prime-labeled and used to probe a human multiple tissue mRNA blot (Clontech). The β-actin cDNA was used as an RNA loading control.

Figure 5


Heart
Brain
Placenta
Lung
Liver
Skeletal muscle
Kidney
Pancreas
Kb
4.4
2.4
0.6
hMIP
β-actin

The ERRT-24 (mitochondrial control region) transcript is estrogen-inducible in breast cancer cells.

The next objective was to study the ER regulation of ERRT-24, a transcript that displayed estrogen-inducibility in the original ddPCR analysis. To confirm these observations, ERRT-24 was used to probe northern blots of total RNA from MCF-7 cells that had been administered 17β-estradiol or ICI 182,780 for six hours. Interestingly, under these conditions, two transcripts were detected, one greater than 9 kb and one smaller than 0.6 kb in molecular weight (Fig. 6). The expression of the high molecular weight transcript was enhanced greater than 5-fold in the RNA population from cells treated with estradiol compared to the ICI 182,780 group, and the smaller transcript was induced 2-fold. In further studies it was noted that ERRT-24 expression was significantly upregulated by estradiol compared to either vehicle or antiestrogen treatment (data not shown). Sequence analysis revealed that ERRT-24 was identical to the mitochondrial control region within the mitochondrial genome. Previously, it has been determined that the entire mitochondrial genome is transcribed as a single multigenic message. The full transcript is later processed into individual genes, many of which code for tRNAs, rRNAs, cytochrome oxidase subunits, and ATPases. The mitochondrial control region is a noncoding 330 bp sequence that is processed in parallel with the other transcripts, and which regulates transcription and replication of the coding strand of the genome. It is likely that in these studies, the high molecular weight message encodes the entire mitochondrial genome and that the small message corresponds to the processed control region. While it is not clear why the high molecular message was upregulated to a greater extent than the smaller RNA, it is

possible that the unprocessed transcript is more stable. Importantly, these results indicate that estrogen, through upregulation of the mitochondrial transcript, augments the expression of all the mitochondrial-encoded genes. This is consistent with the results of others showing that estrogen enhances overall mitochondrial function (Brignone *et al.*, 1987).

Figure 6. The ERRT-24 (mitochondrial control region) transcript is estrogen-inducible in breast cancer cells.

MCF-7 cells were treated for six hours with 50μM cycloheximide and either 10^{-7} M ICI 182,780 (ICI), or 10^{-7} M 17β-estradiol (E2), and total RNA was collected. Equal amounts of total RNA (20 μg) from each treatment group was separated on denaturing gels and transferred to a nitrocellulose membrane. The blot was probed with the random-prime-labeled ERRT-24 (mitochondrial control region) cDNA. The same blot was stripped and reprobed with the 36B4 ribosomal protein (36B4) cDNA to control for equal RNA loading in each lane. The ERRT-24 probe hybridizes with two RNA species, one high molecular weight RNA (> 9 kb) and one low molecular weight RNA (< 0.6 kb).

Figure 6

Body

Part II: The Estrogen Receptor β-Isoform (ERβ) of the Human Estrogen Receptor Modulates ERα Transcriptional Activity and is a Key Regulator of the Cellular Response to Estrogens and Antiestrogens

(please see Appendices, Part A)

Part III: Development of Peptide Antagonists That Target Estrogen Receptor β-Coactivator Interactions

(please see Appendices, Part B)

Key Research Accomplishments

- Identification of two mitochondrial genes regulated by estrogen in human breast cancer cells
- Characterization of the transcriptional activity of the novel human estrogen receptor β (ERβ) subtype of the human estrogen receptor and discovery that the transcriptional activities and mechanisms of the two forms of the human estrogen receptor (ERα and ERβ) are unique
- Discovery that ERβ can function as a transdominant repressor of ERα transcriptional activity at physiological levels of estrogen
- Discovery that ERα and ERβ form heterodimers in mammalian cells, and that the activity of the ERα/ERβ heterodimer is unique from that of either ERα or ERβ alone
- Identification of ERβ-specific interacting peptides that function as ERβ-specific antagonists in mammalian cells

Reportable Outcomes

1. **Hall, J.M.**, Chang, C.-Y. and McDonnell, D.P. 2000. Development of peptide antagonists which target estrogen receptor β-coactivator interactions. Keystone Symposium: Nuclear Receptors 2000, Steamboat Springs, CO.

2. **Hall, J.M.** and McDonnell, D.P. 2000. The human estrogen receptors alpha (ERα) and beta (ERβ): two mechanistically distinct transcription factors (manuscript in preparation).

3. **Hall, J.M.**, Chang, C.-Y. and McDonnell, D.P. 2000. Development of peptide antagonists which target estrogen receptor β-coactivator interactions (*Molecular Endocrinology*, in press).

4. **Hall, J.M**. and McDonnell, D.P. 1999. The estrogen receptor β-isoform (ERβ) of the human estrogen receptor modulates ERα transcriptional activity and is a key regulator of the cellular response to estrogens and antiestrogens. *Endocrinology* **140**, 5566-5578.

5. Gaido, K.W., Leonard, L.S., Maness, S.C., **Hall, J.M.**, McDonnell, D.P., Seville, B.and Safe, S. 1999. Differential interaction of the methoxychlor metabolite 2,2-bis-(p-hydroxyphenyl)-1,1,1-trichloroethane with estrogen receptors α and β. *Endocrinology* **140**, 5746-5753.

6. **Hall, J.M.** and McDonnell, D.P. 1998. Analysis of the molecular pharmacologies of the human estrogen receptors alpha and beta reveals differences in their mechanisms of action. Xth International Congress on Hormonal Steroids, Quebec City, Canada.

7. The U.S. Army Medical Research and Materiel Command under DAMD17-97-1-7277 has supported the Ph.D. training of the principal investigator (degree was conferred on 5/14/00).

8. The graduate work funded by U.S. Army Medical Research and Materiel Command under DAMD17-97-1-7277, has led to the acceptance of Julianne Hall into the SPIRE postdoctoral program (funded by the National Institute of Health) through the University of North Carolina at Chapel Hill.

Conclusions

Part I: Identification of Surrogate Markers of Estrogen Action in Human Breast Cancer Cells

Identification of mitochondrial enzymes as ER targets in breast cancer cells

The most important outcome of this study was the discovery of two novel estrogen regulated target genes, hMIP and the human mitochondrial genome control region. The discovery that the expression of mitochondrial-associated proteins is enhanced by estrogen is consistent with previous findings. Specifically, it has been shown that estrogen enhances cytochrome oxidase activity, augments mitochondrial protein synthesis, and upregulates the mRNA levels of mitochondrially encoded cytochrome oxidase subunits II, and III (Brignone *et al.*, 1987; Van Itallie and Dannies, 1988; Bettini and Maggi, 1992). There is also precedence for estrogen-induced stimulation of mitochondrial function in cancer. Pasqualini *et al.* (1986) observed that administration of ER agonists led to a significant increase in mitochondrial size in the R-27 ER-positive mammary cancer cell line. Furthermore, it was observed that mouse mammary epithelium contains large numbers of mitochondria, which increase in both size and abundance as a consequence of estrogen treatment (Matsumoto *et al.*, 1992). Thus, it is possible that one mechanism by which estrogen displays its mitogenic effects in tumors of the breast and other tissues is through stimulation of mitochondrial function, leading to an increase in cellular energy production which would facilitate cell growth and proliferation. Both MIP and the mitochondrial control region are important estrogen targets in this regard, because the consequences of their upregulation is enhanced activity

and expression of several metabolic enzymes in addition to an overall increase in mitochondrial growth and function. Therefore, it will be important to determine whether the levels of MIP and mitochondrially expressed proteins are enhanced in mammary tumors compared to normal breast epithelial tissue, and to study their regulation in hormone-resistant cancer cells. Furthermore, since ERα is the predominant ER subtype in MCF-7 cells, it will be interesting to examine the role of ERβ in expression of these genes, and in overall mitochondrial function.

The observation that the activated ER is a nuclear transcription factor raises the question as to how estrogen can enhance gene expression and overall function within the mitochondria. In this study, ER regulation of MIP is reasonable based on the observation that the enzyme is encoded within the nuclear genome (Chew *et al.*, 1997). However, the mitochondrial genome seems an unlikely target for ER action in light of the fact that the receptor is not classically thought to associate with cytoplasmic organelles. One possibility is that estrogen induces the expression and/or activation of a factor that is capable of binding within the control region and promoting transcription of the mitochondrial genome. However, no such factor has yet been discovered, and in the current study, the use of the protein synthesis inhibitor cycloheximide indicates that any ER regulated genes identified should have been direct ER targets. Interestingly, recent evidence has emerged that mitochondrial genes are sites for direct action of steroid hormones (Demonacos *et al.*, 1996). Specifically, it was noted that the human mitochondrial genome has sequences resembling HREs, and that the glucocorticoid receptor (GR) and thyroid hormone receptor (TR) are capable of binding to these sites. Furthermore, GR and TR rapidly translocate to the mitochondria following agonist

administration. The most conclusive evidence for hormonal regulation of the mitochondrial genome was the observation that the putative mitochondrial HREs conferred GR and TR agonist inducibility when incorporated into a reporter construct in transient transfection studies, a result that that was abolished in the presence of receptor antagonists. Therefore, it is possible that the mitochondrial genome also contains estrogen responsive enhancers, which would allow for direct ER regulation of mitochondrial gene transcription as suggested by the current study.

ER and RNA stability

The observation that ER ligands enhance the expression of the unprocessed hMIP transcript hints at a role for the receptor in RNA stability. The ability of estrogen to regulate the stability of the hepatic *Xenopus laevis* vitellogenin gene message has been well documented (McKenzie and Knowland, 1990; Dodson and Shapiro, 1994). However, this phenomenon is thought to occur as the consequence of hormone-mediated induction of a protein that binds within the 3' untranslated region of the mRNA. In contrast, the use of cycloheximide in the current study excludes this as a mechanism for the regulation of hMIP expression. Furthermore, hMIP mRNA levels were not enhanced under the conditions tested. Interestingly, ovalbumin gene expression is enhanced by estrogen through a mechanism that involves both RNA stabilization and direct transcriptional effects (Skoufos and Sanders, 1992). Analysis of the DNA structure of the ovalbumin gene revealed that it consists of structural sequences separated by nonintervening sequences, which together, are transcribed to entirety (Roop *et al.*, 1978). Multiple ovalbumin messages are detected in nuclear RNA gels, some of which are greater than four times the size of the processed mRNA species. Furthermore,

accumulation of RNAs corresponding to both structural and intervening sequences was observed during acute estrogen stimulation. Therefore, it is possible that as seen for the ovalbumin gene, hMIP pre-mRNAs are stabilized and/or enhanced by hormone treatment. It is possible that six hours of induction was not sufficient time for processing of this gene, which would account for the absence of an observed estrogen effect on hMIP mRNA levels. Thus, it will be important to study the ER regulation of the 2.4 kb transcript during longer periods of ligand administration. Alternatively, the discrepancy viewed in ER regulation of the hMIP unprocessed RNA versus the mRNA may have been due to the influence of other cellular signaling pathways, as ovalbumin mRNA expression was shown to be decreased by protein kinase C (PKC) activity (Skoufos and Sanders, 1992).

Estrogen and antiestrogen regulation of hMIP

One the most surprising findings of this study was that hMIP is upregulated by both the agonist estradiol and the antagonist ICI 182,780. In view of the fact that these ligands oppose one another's actions both *in vitro* and *in vivo*, it is difficult to understand why they would function similarly in hMIP gene regulation. However, since estradiol and ICI 182,780 display opposite effects in the regulation of many known genes, there are two possible explanations for the results observed in the current study; (1) ICI 182,780 simultaneously induces other genes that neutralize the consequences of hMIP upregulation, or (2) estradiol, but not ICI 182,780, induces intracellular targets of hMIP action, so that only agonist upregulation of hMIP would result in physiological consequences. The observation that estrogen enhances the expression of several mitochondrial enzymes (including hMIP targets) supports the second hypothesis.

Regardless, in view of the fact that hMIP is likely a direct ER target, it will be interesting to characterize the promoter elements involved in estrogen and antiestrogen responses in order to determine whether they occur through similar or distinct mechanisms.

Part II: The Estrogen Receptor β-Isoform (ERβ) of the Human Estrogen Receptor Modulates ERα Transcriptional Activity and is a Key Regulator of the Cellular Response to Estrogens and Antiestrogens

In the second part of my studies (please see Appendices), the aim was to elucidate the molecular mechanisms of action of the human estrogen receptor beta subtype. Overall, these studies show that ERα and ERβ are completely mechanistically distinct transcription factors and that each ER subtype contributes in a unique manner to the overall cellular response to estrogens and antiestrogens. It is now clear that the impact of ERβ on estrogen biology occurs as a consequence of (1) direct actions of ERβ, where it is responsible for regulating target gene transcription and (2) indirect activities, where ERβ modulates ERα action in tissues where they are coexpressed. These observations indicate that the biological responses to estrogens and antiestrogens will be dependent on the relative levels of the two ER subtypes in particular target tissues. The discovery that ERα and ERβ have unique cellular roles indicates that it will be important to develop agents that can selectively target the transcriptional activities of each receptor. While ER subtype selective modulators have not been previously available, the ERβ-specific LXXLL-containing peptides identified in this study (Appendices, Part B) function as highly efficacious ERβ antagonists. These peptides can be used in the future as a tool to study the biological roles of the two ER subtypes.

Importantly, these studies reveal that one biological role of ERβ is to dampen responses to estrogens in cells coexpressing both ER subtypes. It is interesting to note that multiple isoforms of ERβ may contribute to these inhibitory effects. These current studies show that the most widely characterized ERβ isoform, ERβ1, functions as an inhibitor of ERα activity when hormone is limiting (please see Appendices, Part A). To complement these findings, a novel ERβ isoform (ERβ2) was recently described which is deficient in hormone binding activity and as a consequence functions as a dominant negative regulator of ERα and ERβ1 activity (Petersen *et al.*, 1998; Hanstein *et al.*, 1999). Furthermore, Ogawa *et al.* (1998) have also reported the cloning of hERβ cx, an ERβ isoform that functions as a constitutive transrepressor of ERα activity. The existence of several isoforms of ERβ with inhibitory activities suggests that a major role of ERβ is to function as a negative regulator of estrogen action. This inhibitory activity may serve as a mechanism by which some tissues cope with overstimulation by excess estrogens. It will be interesting, therefore, to determine whether the ERβ status of ER-positive breast, ovarian, and endometrial tumor cells is inversely related to their ability to display estrogen-stimulated proliferation. In addition, the observation that ERβ1 dampens responses to estrogens at limiting hormone concentrations and complements ERα activation of estrogen responsive promoters at higher hormone levels, suggests that another role of the receptor is to create a greater window of induction for ER target genes. Therefore, it is likely that in addition to their inhibitory activities, some ERβ isoforms may facilitate rapid biological responses to estrogen during physiological peaks in hormone levels.

Investigation of the mechanisms of ERβ-mediated transrepression

One of the key observations made in this series of studies is that ERβ is constitutively bound to its response element. This result indicates that one mechanism by which ERβ inhibits ERα transcriptional activity is through competition for DNA sites. The forthcoming generation of ERβ DNA binding mutants will permit a direct evaluation of whether the DNA binding function of the receptor is required for its inhibitory effects on ERα activity. The studies performed, however, have not eliminated the possibility ERβ may negatively regulate ERα activity in some contexts by competing for a limiting pool of coactivators that are required for the transcriptional activity of both receptors. In support of this hypothesis, transcription interference experiments showed that ERβ also functions as an inhibitor of PR activity (unpublished data). An evaluation of the ability of ERβ coactivator binding pocket mutants to function as transrepressors of ERα and PR transcriptional activity will address this question, and furthermore, may also indicate whether ERβ-mediated transrepression of ERα and PR occur through similar or distinct mechanisms.

Part III: Development of Peptide Antagonists That Target Estrogen Receptor β-Coactivator Interactions

One of the most significant outcomes of this series of studies is the development of ERβ-specific peptide antagonists (Appendices, Part B). One immediate application of the peptide antagonists is to use them to study the biological role of ERβ in mammalian cells. The peptides could be placed under the control of an inducible promoter in cell lines that contain endogenous ERβ, for example, ovarian granulosa cells and PC12 cells.

These studies would enable us to determine some of the cellular processes regulated by ERβ in the ovary and the central nervous system. Likewise, expression of peptide inhibitors in human breast cancer cell lines expressing both ER subtypes would permit us to determine the contribution of ERβ to estrogen-stimulated cellular proliferation.

It will also be interesting to determine whether the ERβ specific antagonists can be used to modify ER-associated biological effects *in vivo*. Viral administration of peptide to animals under tissue-specific promoters could be used to target particular ER-associated biological responses. For example, in view of the observation that both ER subtypes are expressed in bone tissue, administration of peptide antagonists would permit an assessment of the relative contribution of ERβ to estrogen-mediated maintenance of skeletal integrity. It would be particularly interesting to use these reagents to study ERβ action in tissues expressing both receptor forms, where there is now preliminary evidence that the actions of the two subtypes may oppose each other. For example, it was recently reported that ERβ knockout mice display an increase in bone density (Windahl *et al.*, 1999), exhibit hyperplasia of the prostate and bladder (Krege *et al.*, 1998), and display uterine hyperplasia (Weihua *et al.*, 2000). Interestingly, these phenotypic changes have been observed in tissues where ERα and ERβ colocalize. It remains controversial, however, whether ERβ does in fact function as an antiproliferative receptor *in vivo*, as no such phenotypic changes were observed in mice from the ERβ knockout mouse colony of the Korach group (K. Korach, pers. comm.). Regardless, the ability to target ERβ activity in the same tissues of intact animals with the specific peptide antagonists would enable us to determine whether ERβ inhibits ERα action and/or other biological processes involved in the growth of these tissues, which would help resolve the

controversy over the role of ERβ in these tissues. On this note it will be interesting to introduce the ERβ-specific antagonists into some of the current mouse models of breast cancer in order to determine the contribution of ERβ to estrogen-stimulated mitogenesis in normal and neoplastic breast tissue as well as cell proliferation in diverse tissues.

It has recently been shown that both ER subtypes are expressed in breast tumors and that ERβ expression is upregulated in tumors that have developed tamoxifen resistance (Leygue *et al.*, 1998; Spiers *et al.*, 1999a, b). Thus, there is an unmet medical need to develop novel ER antagonists as (a) potential breast cancer therapeutics and (b) tools to specifically define the role of ERβ in breast cancer cell biology. The finding that none of the LXXLL-containing sequences in this study interact with antiestrogen-liganded receptor suggests that suitably formulated ER peptide antagonists could be coadministered with tamoxifen in order to completely block estrogen-stimulated proliferative pathways in the breast, using two mechanistically distinct modes of antagonism. Recent studies provide evidence that tamoxifen resistance in breast tumors may arise from the upregulation of coactivator proteins, which may permit cells to recognize tamoxifen as an agonist and growth-stimulant (Lavinsky *et al.*, 1998). The identification of peptides that disrupt receptor-coactivator interactions provides a novel mechanism by which the mitogenic actions of activated ER can be blocked in both antiestrogen-responsive and resistant breast cancer cells. Theoretically, the peptide antagonists that we have identified could be developed as second line pharmaceutical treatments for ER-positive, tamoxifen-refractory tumors.

References

Bettini, E., and Maggi, A. (1992). Estrogen induction of cytochrome c oxidase subunit III in rat hippocampus. J. Neurochem. *58*, 1923-1929.

Branda, S.S., and Isaya, G. (1995). Prediction and identification of new natural substrates of the yeast mitochondrial intermediate peptidase. J. Biol. Chem. *270*, 27366-27373.

Brignone, J.A., Ricci, C.R., Brignone, C.M.d., Labonia, N.A., and Stoppani, A.O. (1987). Modulation of D-3-hydroxybutyrate dehydrogenase activity by estrogens. Biochem. Int. *14*, 605-616.

Brown, A., Jeltsch, J., Roberts, M., and Chambon, P. (1984). Activation of pS2 gene transcription is a primary response to estrogen in the human breast cancer cell line MCF- 7. Proc. Natl. Acad. Sci. USA *81*, 6344-6348..

Chew, A., Buck, E.A., Peretz, S., Sirugo, G., Rinaldo, P., and Isaya, G. (1997). Cloning, expression, and chromosomal assignment of the human mitochondrial intermediate peptidase gene (MIPEP). Genomics *4*, 493-496.

Demonacos, C.V., Karayanni, N., Hatzoglou, E., Tsiriyiotis, C., Spandidos, D.A., and Sekeris, C.E. (1996). Mitochondrial genes as sites of primary action of steroid hormones. Steroids *61*, 226-232.

Dodson, R.E., and Shapiro, D.J. (1994). An estrogen-inducible protein binds specifically to a sequence in the 3' untranslated region of estrogen-stabilized vitellogenin mRNA. Mol. Cell. Biol. *14*, 3130-3138.

Dotzlaw, H., Leygue, E., Watson, P.H., and Murphy, L.C. (1996). Expression of estrogen receptor beta in human breast tumors. J. Clin. Endo. Metab. *82*, 2371-2374.

Dotzlaw, H., Leygue, E., Watson, P.H., and Murphy, L.C. (1999). Estrogen receptor-β messenger RNA expression in human breast tumor biopsies: relationship to steroid receptor status and regulation by progestins. Cancer Res. *59*, 529-532.

Gottardis, M.M., and Jordan, V.C. (1988a). Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. Cancer Res. *48*, 5183-5187.

Gottardis, M.M., Robinson, S.P., and Jordan, V.C. (1988b). Estradiol-stimulated growth of MCF-7 tumors implanted in athymic nude mice: a model to study the tumoristatic action of tamoxifen. J. Steroid. Biochem. *30*, 311-314.

Gottardis, M.M., Jiang, S., Jeng, M., and Jordan, V.C. (1989). Inhibition of tamoxifen-stimulated growth of an MCF-7 tumor variant in athymic mice by novel steroidal antiestrogens. Cancer Res. *49*, 4090-4093.

Greene, G.L. (1997). Estrogen receptor modulators and targets. Proceedings 1997. Frontiers in Estrogen Action Conference, Key West, FL.

Hanstein, B., Liu, H., Yancisin, M.C., and Brown, M. (1999). Functional analysis of a novel estrogen receptor-β isoform. Mol. Endocrinol. *13*, 129-137.

Jordan, V.C., and Morrow, M. (1999). Tamoxifen, raloxifene, and the prevention of breast cancer. Endocrine Rev. *20*, 253-278.

Krege, J.H., Hodgin, J.B., Couse, J.F., Enmark, E., Warner, M., Mahler, J.F., Sar, M., Korach, K.S., Gustafsson, J.-A., and Smithies, O. (1998). Generation and reproductive phenotypes of mice lacking estrogen receptor beta. Proc. Natl. Acad. Sci. USA *95*, 15677-15682.

Kuiper, G.G.J.M., Enmark, E., Pelto-Huikko, M., Nilsson, S., and Gustafsson, J.-A. (1996). Cloning of a novel estrogen receptor expressed in rat prostate and ovary. Proc. Natl. Acad. Sci. USA *93*, 5925-5930.

Lavinsky, R.M., Jepsen, K., Heinzel, T., Torchia, J., Mullen, T.-M., Schiff, R., Del-Rio, A.L., Ricote, M., Ngo, S., Gemsch, J., Hilsenbeck, S.G., Osborne, C.K., Glass, C.K., Rosenfeld, M.G., and Rose, D.W. (1998). Diverse signaling pathways modulate nuclear receptor recruitment of N-CoR and SMRT complexes. Proc. Natl. Acad. Sci. USA *95*, 2920-2925.

Leygue, E., Dotzlaw, H., Watson, P.H., and Murphy, L.C. (1998). Altered estrogen receptor α and β messenger RNA expression during human breast tumorigenesis. Cancer Res. *58*, 3197-3201.

Liang, P., and Pardee, A.B. (1992). Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science *257*, 967-971.

Love, R.R., and Koroltchouk, V. (1993). Tamoxifen therapy in breast cancer control worldwide. Bull. World Health Organ. *71*, 795-803.

Marshall, E. (1995). Reanalysis confirms results of tainted study. Science *270*, 1562.

Matsumoto, M., Nishinakagawa, H., Kurohmaru, M., Hayashi, Y., and Otsuka, J. (1992). Effects of estrogen and progesterone on the development of the mammary gland and the associated blood vessels in ovariectomized mice. J. Vet. Med. Sci. *54*, 1117-11124.

McKenzie, E.A., and Knowland, J. (1990). High concentrations of estrogen stabilize vitellogenin mRNA against cytoplasmic degradation but physiological concentrations do not. Mol. Endocrinol. *4*, 807-811.

Morrow, M., and Jordan, V.C. (1993). Molecular mechanisms of resistance to tamoxifen therapy in breast cancer. Arch. Surg. *128*, 1187-1191.

Mosselman, S., Polman, J., and Dijkema, R. (1996). ERβ: identification and characterization of a novel human estrogen receptor. FEBS Let. *392*, 49-53.

Norris, J.D., Fan, D., Wagner, B.L., and McDonnell, D.P. (1996). Identification of the sequences within the human C3 promoter required for estrogen responsiveness provides insight into the mechanism of tamoxifen mixed agonist activity. Mol. Endocrinol. *10*, 1605-1616.

Ogawa, S., Inoue, S., Watanabe, T., Orimo, A., Hosoi, T., Ouchi, T., and Muramatsu, M. (1998). Molecular cloning and characterization of human estrogen receptor β cx: a potential inhibitor of estrogen action in humans. Nucleic Acids Res. *26*, 3505-3512.

Pasqualini, J.R., Giambiagi, N., Sumida, C., Nguyen, B.L., Gelly, C., Mayrand, C., and Lecerf, F. (1986). Biological responses of tamoxifen in the fetal and newborn vagina and uterus of the guinea-pig and in the R-27 mammary cancer cell line. J. Steroid Biochem. *24*, 99-108.

Petersen, D.N., Tkalcevic, G.T., Koza-Taylor, P.H., Turi, T.G., and Brown, T.A. (1998). Identification of estrogen receptor beta2, a functional variant of estrogen receptor beta expressed in normal rat tissues. Endocrinology *139*, 1082-1092.

Roop, D.R., Nordstrom, J.L., Tsai, S.Y., Tsai, M.J., and O'Malley, B.W. (1978). Transcription of structural and intervening sequences in the ovalbumin gene and identification of potential ovalbumin mRNA precursors. Cell *15*, 671-685.

Skoufos, E., and Sanders, M.M. (1992). Regulation of expression of the chicken ovalbumin gene: interactions between steroid hormones and second messengers. Mol. Endocrinol. *6*, 1412-1417.

Smith, C.L. (1998). Cross-talk between peptide growth factor and estrogen receptor signaling pathways. Biol. Reprod. *58*, 627-632.

Speirs, V.C., Malone, C., Walton, D.S., Kerin, M.J., and Atkin, S.L. (1999a). Increased expression of estrogen receptor β mRNA in tamoxifen-resistant breast cancer patients. Cancer Res. *59*, 5421-5424.

Speirs, V.C., Parkes, A.T., Kerin, M.J., Walton, D.S., Carleton, P.J., Fox, J.N., and Atkin, S.L. (1999b). Coexpression of estrogen receptor α and β: poor prognostic factors in human breast cancer? Cancer Res. *59*, 525-528.

Van Itallie, C.M., and Dannies, P.S. (1988). Estrogen induces accumulation of the mitochondrial ribonucleic acid for subunit II of cytochrome oxidase in pituitary tumor cells. Mol. Endocrinol. *4*, 332-337.

Weihua Z., Saji S., Makinen S., Cheng G., Jensen E.V., Warner M., and Gustafsson J.-A. (2000). Estrogen receptor (ER) β, a modulator of ERα in the uterus. Proc. Natl. Acad. Sci. USA *97*:5936-5941.

Willson, T.M., Norris, J.D., Wagner, B.L., Asplin, I., Baer, P., Brown, H.R., Jones, S.A., Henke, B., Sauls, H., Wolfe, S., Morris, D.C., and McDonnell, D.P. (1997). Dissection of the molecular mechanism of action of GW5638, a novel estrogen receptor ligand, provides insights into the role of ER in bone. Endocrinology *138*, 3901-3911.

Windahl, S.H., Vidal, O., Andersson, G., Gustafsson, J.-A., and Ohlsson, C. (1999). Increased cortical bone mineral content but unchanged trabecular bone mineral density in female ERbeta (-/-) mice. J. Clin. Invest. *104*, 895-901.

Wittliff, J.L. (1984). Steroid-hormone receptors in breast cancer. Cancer *53*, 630-643.

Appendices

Appendix A:

The Estrogen Receptor β-Isoform (ERβ) of the Human Estrogen Receptor Modulates ERα Transcriptional Activity and is a Key Regulator of the Cellular Response to Estrogens and Antiestrogens

(please see attached manuscript- pgs. 50-62)

Appendix B:

Development of Peptide Antagonists That Target Estrogen Receptor β-Coactivator Interactions

(please see attached manuscript- pgs. 63-76)

0013-7227/99/$03.00/0
Endocrinology

Vol. 140, No. 12
Printed in U.S.A.

The Estrogen Receptor β-Isoform (ERβ) of the Human Estrogen Receptor Modulates ERα Transcriptional Activity and Is a Key Regulator of the Cellular Response to Estrogens and Antiestrogens*

JULIE M. HALL† AND DONALD P. McDONNELL

Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, North Carolina 27710

ABSTRACT

The human estrogen receptor α (ERα) and the recently identified ERβ share a high degree of amino acid homology; however, there are significant differences in regions of these receptors that would be expected to influence transcriptional activity. Consequently, we compared the mechanism(s) by which these receptors regulate target gene transcription, and evaluated the cellular consequences of coexpression of both ER subtypes. Previously, it has been determined that ERα contains two distinct activation domains, ERα-AF-1 and ERα-AF-2, whose transcriptional activity is influenced by cell and promoter context. We determined that ERβ, like ERα, contains a functional AF-2, however, the ERβ-AF-2 domain functions independently within the receptor. Of additional significance was the finding that ERβ does not contain a strong AF-1 within its amino-terminus but, rather, contains a repressor domain that when removed, increases the overall transcriptional activity of the receptor. The importance of these findings was revealed when it was determined that ERβ functions as a transdominant inhibitor of ERα transcriptional activity at subsaturating hormone levels and that ERβ decreases overall cellular sensitivity to estradiol. Additionally, the partial agonist activity of tamoxifen manifest through ERα in some contexts was completely abolished upon coexpression of ERβ. In probing the mechanisms underlying ERβ-mediated repression of ERα transcriptional activity we have determined that 1) ERα and ERβ can form heterodimers within target cells; and 2) ERβ interacts with target gene promoters in a ligand-independent manner. Cumulatively, these data indicate that one role of ERβ is to modulate ERα transcriptional activity, and thus the relative expression level of the two isoforms will be a key determinant of cellular responses to agonists and antagonists. (*Endocrinology* **140:** 5566–5578, 1999)

THE HUMAN ESTROGEN receptor (ER) belongs to the nuclear receptor superfamily of ligand-inducible transcription factors (1), whose members include the receptors for steroids, thyroid hormone, retinoic acid, vitamin D, and orphan receptors for which no ligands have yet been identified. The mechanism of action of ER is similar to that of other nuclear receptors. In the absence of hormone, the receptor is sequestered within the nuclei of target cells in a multiprotein inhibitory complex. The binding of ligand induces an activating conformational change within ER, an event that promotes homodimerization and high affinity binding to specific DNA response elements (EREs) located within the regulatory regions of target genes (2). In addition to the classic ligand-mediated activation pathway, it has been shown that ER can be activated in the absence of ligand by growth factors or other agents that elevate intracellular cAMP levels (3, 4). Although the physiological importance of the ligand-independent signaling pathways remains to be determined, it has been shown in ER knockout mice that the uterotropic responses to both 17β-estradiol and epidermal growth factor require a functional ER (5). Thus, ER appears to be a key point of convergence of multiple signaling pathways, an observation that complicates our understanding of the pharmacology of estrogens and antiestrogens.

Until recently it was considered that a single ER was responsible for all of the biological actions of estrogens and antiestrogens. However, the recent identification of ERβ (6, 7) has indicated that the cellular responses to ER ligands are far more complex. The two estrogen receptors, ERα and ERβ, have similar overall structures, displaying a high degree of amino acid conservation in the central DNA-binding domain (DBD) and moderate conservation in the ligand-binding domain (LBD; C-terminus), but considerable divergence in the amino-terminus. Not surprising, therefore, ERα and ERβ interact with the same DNA response elements (8) and exhibit similar, but not identical, ligand binding characteristics. Although a specific physiological role for ERβ remains to be defined, its identification has provided a potential explanation for the biological actions of estrogen(s) in cells where no immunoreactive ERα could be detected. Interestingly, preliminary localization studies have revealed that there are many tissues in which both ER subtypes are coexpressed (6, 7, 9). Thus, the impact of ERβ on estrogen biology is likely to occur as a consequence of 1) direct actions of ERβ, where it is responsible for regulating target gene transcription; and 2) indirect activities, where ERβ modulates ERα action in tissues where they are coexpressed.

Although the precise mechanism by which ER regulates transcription remains to be determined, considerable

Received April 5, 1999.
Address all correspondence and requests for reprints to: Dr. Donald P. McDonnell, Department of Pharmacology and Cancer Biology, Duke University Medical Center, P.O. Box 3813, Durham, North Carolina 27710. E-mail: mcdon016@acpub.duke.edu.
* This work was supported by NIH Grant DK-48807 (to D.P.M.).
† Supported by a Predoctoral Fellowship from the U.S. Army Medical Research Acquisition Activity.

progress has been made in defining the domains within ERα required for its activity. Specifically, it has been demonstrated that the transcriptional activity of ERα is mediated by two activation functions (AFs) located in the amino-terminal (AF-1) and carboxyl-terminal (AF-2). Although both of these AFs function in a synergistic manner in most circumstances, they can also function independently in a cell- and promoter-specific manner, an activity that may explain the tissue-selective agonist activity of some ER ligands (10, 11). In this regard, it has been observed that 17β-estradiol can function as an agonist in all environments regardless of whether AF-1 or AF-2 is the dominant activator. Not surprisingly, therefore, the pure antiestrogen ICI 182,780, which inhibits the activity of both AF-1 and AF-2, completely blocks the ability of ERα to activate transcription through classical ERE-mediated pathways. Unlike the pure antiestrogens, however, the relative agonist/antagonist activities of most other antiestrogens are determined by the cell and promoter context. For instance, compounds such as tamoxifen inhibit AF-2 activity, and consequently function as antagonists in all environments where AF-2 is required. In contexts where AF-1 is the dominant activator, on the other hand, tamoxifen manifests partial agonist activity. These observations led to the hypothesis that the tissue-selective biological activity exhibited by selective ER modulators (SERMs) such as tamoxifen, reflects their ability to differentially regulate AF-1 or AF-2. However, the identification of ER ligands such as raloxifene and GW5638, which function as estrogens in the bone and the cardiovascular system but do not appear to function as either AF-1 or AF-2 agonists, indicates that the existing models of ER pharmacology are incomplete (12, 13). Clearly, they must now be expanded to include a consideration of the impact of ERβ. It is likely that the existence of ERα and ERβ will be as important to ER pharmacology as the two progesterone receptor subtypes, PR-A and PR-B, are to the pharmacology of progestins and antiprogestins. We base this hypothesis on our earlier studies of PR action, where it was shown that PR-A and PR-B were not functionally identical. Specifically, we observed that both receptor forms could manifest autonomous activity in some cell contexts, whereas in others the A isoform was a weak transcriptional activator and, in fact, functioned as a transdominant inhibitor of human PR-B activity (14). The possibility that there were similarities between these two systems prompted us to explore the impact of ERβ on the pharmacology of ERα. Although ERα/ERβ are not derived from the same gene as are the two forms of PR, we believed that it would be useful to consider ERα/ERβ as having a similar relationship as PR-A/B. The aim of this study, therefore, was to compare the transcriptional activities of ERα and ERβ and to evaluate the contribution of ERβ to the overall pharmacology of estrogens and antiestrogens.

Materials and Methods

Biochemicals

DNA restriction and modification enzymes were obtained from Roche Molecular Biochemicals (Indianapolis, IN), New England Biolabs, Inc. (Beverly, MA), or Promega Corp. (Madison, WI). PCR reagents were obtained from Perkin Elmer Corp. (Norwalk, CT) or Promega Corp. 17β-Estradiol and 4-hydroxytamoxifen were purchased from Sigma Chemical Co. (St. Louis, MO). The ER antagonist ICI 182,780 was a gift from Dr. Alan Wakeling (Zeneca Pharmaceuticals, Macclesfield, UK). Raloxifene was a gift from Dr. Eric Larsen, Pfizer, Inc. (Groton, CT). GW7604 was a gift from Dr. Tim Willson (Glaxo-Wellcome, Research Triangle Park, NC). Idoxifene was a gift from Dr. Maxine Gowan (SmithKline Beecham, King of Prussia, PA). The mouse monoclonal anti-FLAG antibody was purchased from Sigma Chemical Co. Secondary antibodies, Hybond-C extra transfer membranes, and enhanced chemiluminescence reagents were purchased from Amersham Pharmacia Biotech (Arlington Heights, IL).

Plasmids

The mammalian expression plasmid for ERα (pRST7ER) has been described previously (11). Plasmids expressing ERα mutants (ER-AF1, ER-AF2, ER-Null) were also described previously (11). The ERβ expression plasmid, pRST7ERβ, was constructed as follows. A PCR 3.1 vector (Invitrogen, Carlsbad, CA) containing the human ERβ coding sequence (amino acids 1–477; gift from Dr. Mark Nuttall, SmithKline Beecham) was digested with *Hin*dIII and *Xba*I, and ERβ was ligated into pRST7ER, previously digested with *Hin*dIII and *Xba*I to remove the ERα complementary DNA (cDNA). An epitope-modified series of ERα and ERβ expression vectors was also created. Specifically, an amino-terminus FLAG tag was added to the ERα and ERβ expression vectors by PCR. The sequences of the oligonucleotides for PCR were 5′-GTGGACGTCGACATGGACTACAAAGACGACGACGACAAAATGACCATGACCCTCCAC (forward) and 3′-GTGGAGGGATCCTCAGACTGTGGCAGGGAAACC (reverse) for ERα and 5′-GTGGACGTCGACATGGACTACAAAGACGACGACGACAAAATGAATTACAGCATTCCC (forward) and 3′-GTGAGGTCTAGATCACTGAGACTGTGGGTT (reverse) for ERβ.

The mammalian expression plasmid for the ERβ mutant ERβ-AF1 was constructed by site-directed mutagenesis. The pRST7ERβ plasmid was used as the template, and the three point mutations were created using PCR-based oligonucleotide-directed mutagenesis, according to the manufacturer's protocol (Stratagene, La Jolla, CA). The sequences of the oligonucleotides used for PCR were 5′-GTGGTCCCAGTGTATGCCCTGCTGCTGGCG ATGCTGGCTGCCCACGTGCTTCGCGGG (forward) and 5′-CCCGCGAAGCACGTGGGCAGCCAGCATCGCCAGCAGCAGGGCATACAC TGGGACCAC (reverse).

The plasmid ERβ-AF2 was constructed as follows: an empty pRST7 vector was first created by digesting pRST7ER with *Hin*dIII and *Sma*I to remove the ERα cDNA, blunt ends within the vector were created with Klenow, and the vector was recircularized by ligation. The pRST7 vector was digested with *Xba*I, and a PCR fragment was generated from pRST7ERβ (containing amino acids 90–477 of the coding sequence for ERβ) and ligated into the pRST7 vector. The sequences of the oligonucleotides used for PCR were 5′-GTGAGGTCTAGAATGAAGAGGGATGCTCACTTC (forward) and 3′-GTGAGGTCTAGATCACTGAGACTGTGGGTT (reverse).

To compare the stability of the ERβ mutants in transfected cells, we created a duplicate set of vectors in which an amino-terminus FLAG tag was added to the expression vectors for the ERβ mutants by PCR. The sequences of the oligonucleotides used for PCR for the ERβ-AF1 mutant were the same as those used to construct the FLAG-tagged wild-type ERβ. The oligonucleotides used to create the FLAG-tagged ERβ-AF2 mutant were 5′-GTGGACTCTAGAATGGACTACAAAGACGACGACGACAAATGCGCTGTCTG CAGCGATTAC (forward) and 3′-GTGAGGTCTAGATCACTGAGACTGTGGGTT (reverse).

The GAL4-DBD-ER N-terminus fusion constructs were cloned into the pBK-cytomegalovirus (CMV) mammalian expression vector. Construction of the pBKC-DBD plasmid has been described previously (15). pBKC-DBD-ERα-(1–182) was constructed as follows. The pBKC-DBD plasmid was digested with *Eco*RI and *Cla*I, and a PCR fragment (containing the coding sequence for the first 182 amino acids of ERα) was generated from pRST7ER and ligated into these sites. The sequences of the oligonucleotides for PCR were 5′-GTGCAGGAATTCATGACCATGACCCTCCAC (forward) and 5′-GTGCAGATCGATAGTCTCCTTGGCAGATTC (reverse). pBKC-DBD-ERβ-(1–95) was constructed as follows. The pBKC-DBD plasmid was digested with *Eco*RI and *Cla*I, and a PCR fragment (containing the coding sequence for the first 95 amino acids of ERβ) was generated from pRST7ERβ and ligated into these sites. The sequences of the oligonucleotides for PCR were 5′-GTGCAGGAATTCATGAATTACAGCATTCCC (forward) and 5′-GTGCAGATCGATGAAGTGAGCATCCCTCTT (reverse).

pBKC-DBD-ERα-LBD(3x) was constructed as follows: the pBKC-DBD plasmid was digested with *Eco*RI and *Cla*I, and a PCR fragment (containing the coding sequence for amino acids 282–595 of ERα) was generated from pRST7-ER-AF-1 and ligated into these sites. The sequences of the oligonucleotides for PCR were 5′-GTGCAGGAATTCATGTCTGCTGGAGACATGAGA (forward) and 3′-GTGCAGATCGATGACTGTGGCAGGGAAACC (reverse).

All of the PCR-based constructs were sequenced to verify the accuracy of the amplified sequences.

Cell culture and transient transfection assays

HepG2, HeLa, and 293 cells were maintained in MEM (Life Technologies, Inc.) supplemented with 10% FCS (Life Technologies, Inc.). MCF-7 and SKBR3 cells were maintained in DMEM (Life Technologies, Inc.) supplemented with 10% FCS (Life Technologies, Inc.). Cells were plated in 24-well plates (coated with gelatin for transfections of HepG2 cells) 24 h before transfection. DNA was introduced into the cells using lipofectin (Life Technologies, Inc.). Triplicate transfections were performed using 3 μg total DNA. In standard transfections, 1500 ng reporter (C3-Luc, 3x-ERE-TATA-Luc, or 5x-GAL4-TATA-Luc), 500 ng receptor (pRST7ER, pRST7ERβ, ER mutants, or GAL4 fusions), 100 ng of the pBKC-βgal normalization vector (16), and 900 ng of the control vector pBSII-KS (Stratagene) were used. The reporter C3-Luc contains the estrogen-responsive complement 3 gene promoter, and the 3x-ERE-TATA-Luc reporter contains three copies of the vitellogenin ERE. The reporter 5x-GAL4-TATA-Luc (a gift from Dr. Xiao-Fan Wang, Duke University Medical Center) contains five palindromic copies of the GAL4 transcription factor response element cloned into pGL2-TATA-Inr (Stratagene). Cells were incubated with the DNA/lipofectin mix for 3 h, then washed with PBS and incubated with the appropriate hormone in phenol red-free medium containing 10% charcoal-stripped FCS (HyClone Laboratories, Inc., Logan, UT) for 48 h. Luciferase and β-galactosidase assays were performed as described previously (17). All experiments were repeated a minimum of three times.

Western immunoblot analysis

293 cells (human embryonic kidney cells) were transfected with the expression plasmids for ERα, ERβ, or the ERβ mutants. Whole cell extracts were prepared as described previously (18). Fifty micrograms of whole cell extracts for each sample were run on a 10% SDS-PAGE gel and transferred to nitrocellulose. Immunoblotting was performed using a mouse monoclonal anti-FLAG antibody. Immunocomplexes were detected by ECL.

Results

The α- and β-forms of the human ER are functionally distinct

We used a cotransfection assay reconstituted in HepG2 (human hepatoma) or HeLa (human cervical carcinoma) cells to compare the transcriptional activities and ligand responsiveness of ERα and ERβ. These cell lines were chosen for our studies because they require exogenous ERα or ERβ to activate ERE-mediated transcription, enabling an assessment of the transcriptional responses of each receptor in isolation. Specifically, the ER expression vectors (pRST7ER and pRST7ERβ) were transiently transfected into either HepG2 or HeLa cells together with the estrogen-responsive reporter 3x-ERE-TATA-Luc or C3-Luc. The input concentration for each expression vector used in these studies was equivalent and was that which gave a maximal response under the conditions of the assay. In addition, using a duplicate set of vectors in which we added an amino-terminus FLAG epitope, we could show by Western immunoblot analysis that the ERα and ERβ cDNAs directed similar levels of ER expression (data not shown). Although we demonstrated that the presence of the FLAG tag did not affect the estradiol-mediated transcriptional activity of either receptor (data not shown), we elected to use the native receptors for our studies because we were unsure how the FLAG tag would affect more subtle functions of ERα and ERβ. Using this system we examined the transcriptional responses of ERα and ERβ over a range of concentrations of the different ER ligands (Fig. 1A). Both receptors were activated by 17β-estradiol, although we observed that ERα is a more efficacious activator in this model system. Interestingly, all of the SERMS and pure antagonists tested displayed no agonist or inverse agonist activities on ERβ on either of the promoters studied (Fig. 1A and data not shown). As shown previously (16), 4-hydroxytamoxifen displayed partial agonist activity on ERα in HepG2 cells on the C3-Luc reporter. In this environment, GW7604 did not exhibit agonist activity on ERα, whereas ICI 182,780, raloxifene, and idoxifene functioned as inverse agonists.

The initially reported form of the human ERβ is 477 amino acids in length (ERβ-short), and its expression has been verified in several tissues (7). However, an isoform that contains an additional 53 amino acids at the N-terminus (ERβ-long) has recently been described, and this form is also detectable *in vivo* (19). We characterized the transcriptional activity of the two ERβ isoforms to determine whether the additional sequences had any impact on the observed responsiveness to estrogens and antiestrogens. However, under the conditions of our assays, the activities of ERβ-short or ERβ-long in response to ER agonists and antagonists were indistinguishable (data not shown). Cumulatively, these results suggest that the responses of ERα and ERβ to pharmacological agents are different, a finding that may be important in understanding the cell-selective actions of these compounds.

We observed that 17β-estradiol was a stronger activator of ERα than ERβ in HepG2 cells on the C3-Luc reporter (Fig. 1A). To investigate whether this observation holds in other cell and promoter contexts, we examined the effect of the agonist 17β-estradiol on ERα and ERβ transcriptional activities in HepG2 and HeLa cells on the 3x-ERE-TATA-Luc and C3-Luc reporters (Fig. 1, B–D). In the absence of hormone, ERα showed a significantly higher level of basal transcriptional activity than ERβ in both HepG2 (Fig. 1B) and HeLa cells (Fig. 1, C and D). This effect was observed on both the simple and complex promoters. In comparing the overall efficacies of ERα and ERβ, the basal activity of the former must be considered, as we have shown previously that this activity is receptor-dependent and can be completely suppressed using pure antiestrogens (16). It has been reported previously that ERα and ERβ have equivalent affinities for estradiol (8); however, in our assays the EC_{50} for estradiol was approximately 1.5 orders of magnitude less for ERα than for ERβ in both HepG2 (Fig. 1B) and HeLa (data not shown) cells. Furthermore, the efficacies displayed by ERα were consistently higher than those displayed by ERβ under the conditions we used (Fig. 1, B and C). Reproducibly, we found that ERβ demonstrates about 20–60% of the total activity of ERα. Similar results were observed in transient transfection assays performed in ROS (rat osteosarcoma) and SKBR3 (mammary carcinoma) cell lines (data not shown). Based on these studies, we conclude that 17β-estradiol is a more potent

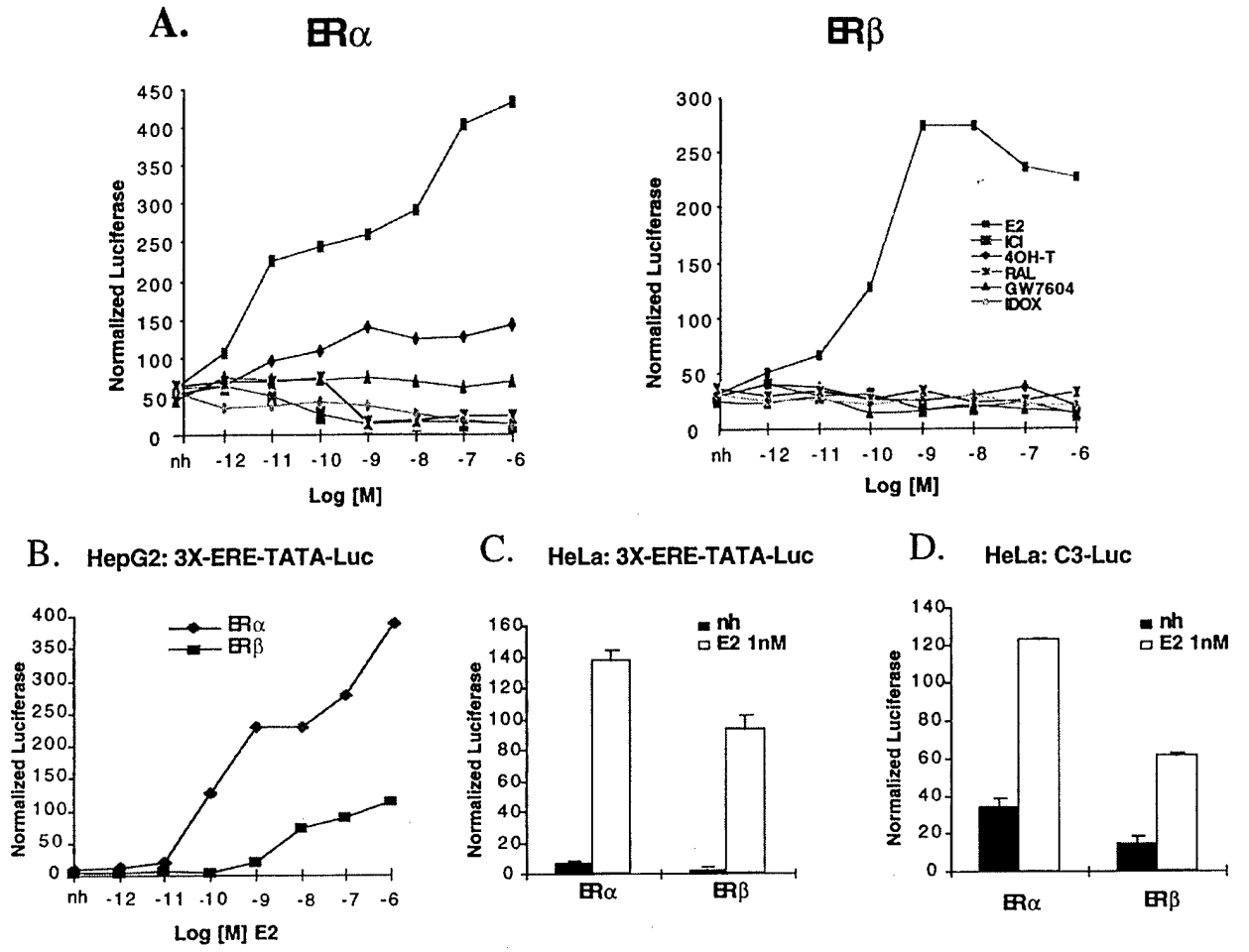


FIG. 1. The α- and β-forms of the human ER are functionally distinct. A, HepG2 cells were transiently transfected with the ERα or ERβ expression vectors and the C3-Luc reporter. Cells were induced with vehicle (nh) or increasing concentrations (ranging from 1 pM to 1 μM) of 17β-estradiol (E2), ICI 182,780 (ICI), 4-hydroxytamoxifen (4OH-T), raloxifene (RAL), GW7604, or idoxifene (IDOX). After 48 h, transcription was quantitated by assaying for luciferase activity, and all transfections were normalized for efficiency using an internal β-galactosidase control plasmid (pCMV-β-gal). Each *data point* is the average of triplicate measurements of transcriptional activity, and the average coefficient of variation of each value is less than 10%. B, HepG2 cells and HeLa cells (C and D) were transfected with the ERα or ERβ expression plasmids and the 3x-ERE-TATA-Luc or C3-Luc reporter. Cells were induced with 17β-estradiol (E2) for 48 h, and luciferase assays were performed. Each value was normalized to the β-galactosidase activity. Each *data point* is the average of triplicate determinations, and the average coefficient of variance for each value is less than 10%.

and efficacious activator of ERα, and that it is likely that both receptors contribute in a unique manner to the cellular response to estrogens. Overall, our results define a major mechanistic distinction between the two ERs; ERβ is strictly dependent on pure agonists for the activation of transcription from its target promoters, whereas ERα can be activated by both agonists, partial agonists (SERMS), and ligand-independent mechanisms.

The activation domains within ERα and ERβ are not functionally equivalent

We and others have shown that both activation domains, AF-1 and AF-2, are required for maximal agonist-dependent and ligand-independent activation of transcription by ERα (11). Additionally, in contexts where ERα-AF-1 alone can function as an autonomous activator we were able to demonstrate that 4-hydroxytamoxifen manifests partial agonist activity. Using similar assays, reconstituted in several cell and promoter backgrounds, we were unable to detect significant ERβ-mediated 4-hydroxytamoxifen agonist activity, suggesting that this receptor isoform may not possess a functional AF-1 or, alternatively, that it may have a different type of activation domain within this region.

To define the mechanism(s) underlying the differential activation profiles of ERα and ERβ, we wanted to determine the relative contributions of the N-terminus (AF-1) and C-terminus (AF-2) activation domains to the transcriptional activity of the whole receptors. Previously, our laboratory has created mutations in ERα that abolish the activity of AF-1 or AF-2 (11). We have now constructed the corresponding

mutations in ERβ (Fig. 2A), and this has enabled us to assess the relative contributions of each AF to the transcriptional activities of ERα and ERβ. To compare the activities of our mutants, HepG2 cells and HeLa cells were transiently transfected with wild-type ERα or ERβ or the mutant receptor to be tested together with the C3-Luc reporter. Because of the difficulty in obtaining antibodies that can be used to measure the relative expression of ERα and ERβ, we elected to per-

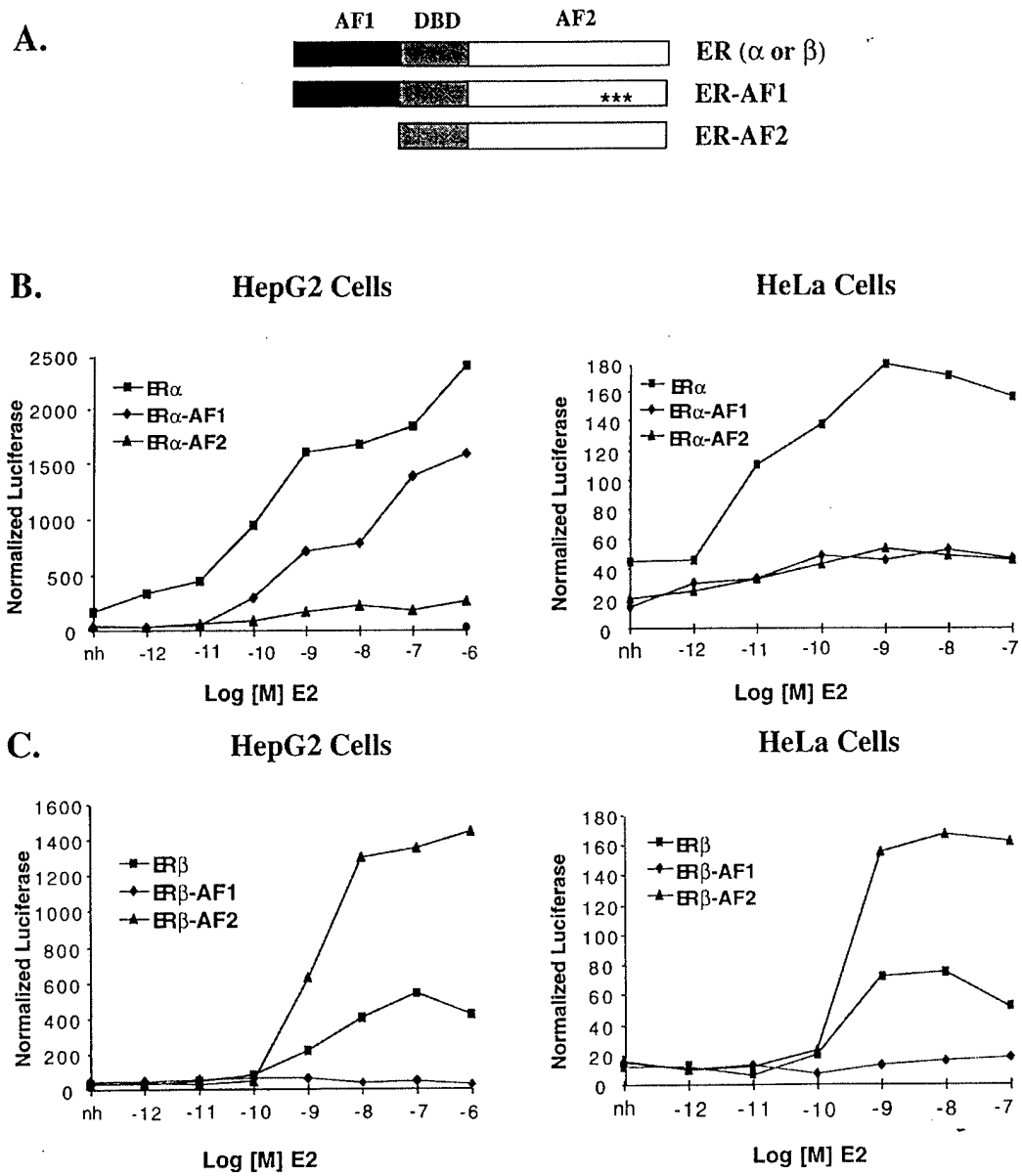


FIG. 2. The activation domains within ERα and ERβ are not functionally equivalent. A, The ERα activation domain mutants were created, as described previously (37). The ERβ-AF1 construct was made by introducing three amino acid changes into the AF2 region of the receptor, substituting alanine for amino acids located at positions 436, 440, and 443. The ERβ-AF2 construct was made by deleting the N-terminus (amino acids 1–95) of the wild-type receptor. B, HepG2 cells and HeLa cells were transiently transfected with the ERα wild-type or mutant receptors together with the C3-Luc reporter. After transfection, cells were treated with vehicle (nh) or increasing concentrations (ranging from 1 pM to 1 μM) of 17β-estradiol (E2). After 48 h, cells were harvested and assayed for luciferase activity, and all transfections were normalized for efficiency using the internal pCMV-β-gal control plasmid. Each *data point* is the average of triplicate determinations, and the average coefficient of variance for each value is less than 10%. C, HepG2 cells and HeLa cells were transiently transfected with the ERβ wild-type or mutant receptor together with the C3-Luc reporter and the pCMV-β-gal control vector. Cells were induced with 17β-estradiol (E2) for 48 h, and luciferase assays were performed. Each value was normalized to the β-galactosidase activity. Each *data point* is the average of triplicate determinations, and the average coefficient of variance for each value is less than 10%.

form all of our studies at input plasmid concentrations that yield the maximal activity in a given assay. Consequently, our studies do not allow us to compare ERα and ERβ on a molecule:molecule basis, but, rather, permit us to compare these receptors at a functional level. We have successfully used this approach in the past to compare the transcriptional activities of a series of ERα mutants (11). The results of this analysis are shown in Fig. 2. As expected, ERα displays a dose-dependent increase in activity in the presence of 17β-estradiol in both HepG2 and HeLa cells (Fig. 2B). In addition, as shown before (37), mutants containing AF-1 or AF-2 alone are also capable of activating transcription, although their activities are influenced by both the cell and promoter context in which they were assayed. In HepG2 cells, for instance, ERα-AF-1 is significantly more active than ERα-AF-2; thus, in this environment AF-1 appears to be the dominant activator. In HeLa cells, however, both ERα-AF-1 and ERα-AF-2 display identical activation profiles, and their combined activity is significantly less than that of the intact receptor throughout the entire range of hormone concentrations. Interestingly, in both cell contexts, ERα-AF-1 and ERα-AF-2 exhibit significantly lower ligand-independent activity compared with the intact ERα. These studies confirm our previous findings that both AF-1 and AF-2 contribute to the overall transcriptional activity of ERα, and that the relative activity of each activation domain is dependent on the cell context.

We next examined the activities of ERβ and the ERβ AF mutants in HepG2 and HeLa cells (Fig. 2C). To find suitable dose ranges of input plasmids for our studies, we created a duplicate set of vectors in which the amino-termini of the mutant receptors were modified by the addition of a FLAG tag. Using these expression vectors we were able to demonstrate that ERβ and ERβ-AF-1 were expressed equivalently, whereas ERβ-AF-2 expression was elevated by about 50%. With this information in hand, we proceeded with the evaluation of the transcriptional activity of the untagged, native ERβ mutants. Because our vectors produce equivalent amounts of receptor, we performed all of our studies at equivalent input plasmid concentrations, selecting that which gave a maximal response under the conditions of the assay for all receptors. As seen with ERα, the wild-type ERβ is a ligand-dependent transcriptional activator in both cell lines. However, although ERα-AF-1 can function autonomously in some cell types, ERβ-AF-1 is transcriptionally inactive in both HepG2 and HeLa cells. Overall, the most surprising finding was that removal of the amino-terminus (ERβ-AF-2) enhances ERβ transcriptional activity rather than decreases it as was seen with ERα. Of additional importance was the finding that ERβ-AF-2 is extremely active in environments where ERα-AF-2 is essentially transcriptionally inactive. Therefore, we conclude that the C-terminus (AF-2) mediates the transcriptional activity of ERβ in response to 17β-estradiol, and that this domain functions independently of the amino-terminus. Furthermore, sequences within the N-terminus have a negative impact on the overall transcriptional activity of ERβ, and their removal creates a mutant whose function is similar, but not identical, to that of ERα. It is possible that ERβ does, in fact, contain a functional AF-1 domain and that it may be functional in contexts not represented by our assays. These studies clearly indicate that the activation domains of ERα and ERβ are not identical and that these two receptors are different transcription factors that likely have distinct roles in estrogen biology.

The amino-terminus of ERα, but not that of ERβ, functions as an autonomous activating sequence

To determine whether the amino-terminus of ERβ does, in fact, possess an autonomous activation function, we evaluated the transcriptional activity of the ERα and ERβ N-termini when removed from the context of their intact receptors. Specifically, the N-termini of ERα and ERβ were each fused to the GAL4 transcription factor DBD (Fig. 3A), and the activity of each construct was compared in transient transfection studies in HepG2, HeLa, MCF-7 (human mammary carcinoma), and SKBR3 (human mammary adenocarcinoma) cells on a GAL4-responsive reporter (Fig. 3, B–E). The input concentration of each vector used in these studies was that which gave a maximal response under the conditions of the assay. In HepG2 cells, the GAL4-DBD-ERα construct possessed more than 18 times the activity of the GAL4 DBD alone, whereas only a 2.4-fold enhancement by the ERβ construct was observed. In HeLa cells, ERα displayed a 5.5-fold increase in transcriptional activity over the control, whereas only a 1.4-fold increase was observed for ERβ. Similarly, in MCF-7 and SKBR3 cells, ERα displayed 42- and 53-fold increases in activities, whereas only 3.3- and 6-fold increases were seen for ERβ. These studies illustrate that while the N-terminus of ERα has a strong activation domain that functions in a cell-specific manner, the homologous region in ERβ is much less active. Therefore, it is likely that the repressor function is the primary determinant of the activity of the N-terminus of ERβ in the whole receptor, and that the distinct transcriptional profiles of the two ERs are mediated in part by differences in their amino-termini.

ERβ represses ERα transcriptional activity at subsaturating concentrations of 17β-estradiol

Our data indicate that ERα and ERβ are not functionally equivalent and that in many contexts ERα is significantly more transcriptionally active than ERβ. Therefore, we considered the possibility that, similar to the effects mediated by PR-A on PR-B activity, ERβ may act as a transdominant repressor or a modulator of ERα activity in contexts where ERβ is less active. To test this hypothesis, we examined the effect of ERβ expression on the transcriptional activity of ERα when both receptors were expressed in target cells. Specifically, the ERα expression vector was transiently transfected into HepG2 cells, either alone or in the presence of increasing amounts of the ERβ expression vector together with the 3x-ERE-TATA-Luc reporter. The cells were induced with either 100 nM 17β-estradiol, a saturating concentration at which both receptors demonstrate maximal transcriptional activity, or 100 pM 17β-estradiol, a subsaturating concentration at which only ERα is active. As expected, ERα was a better activator of transcription than ERβ when the assay was performed in the presence of 100 nM 17β-estradiol. Interestingly, although ERα manifests significant ligand-dependent activity, the introduction of increasing amounts of ERβ into

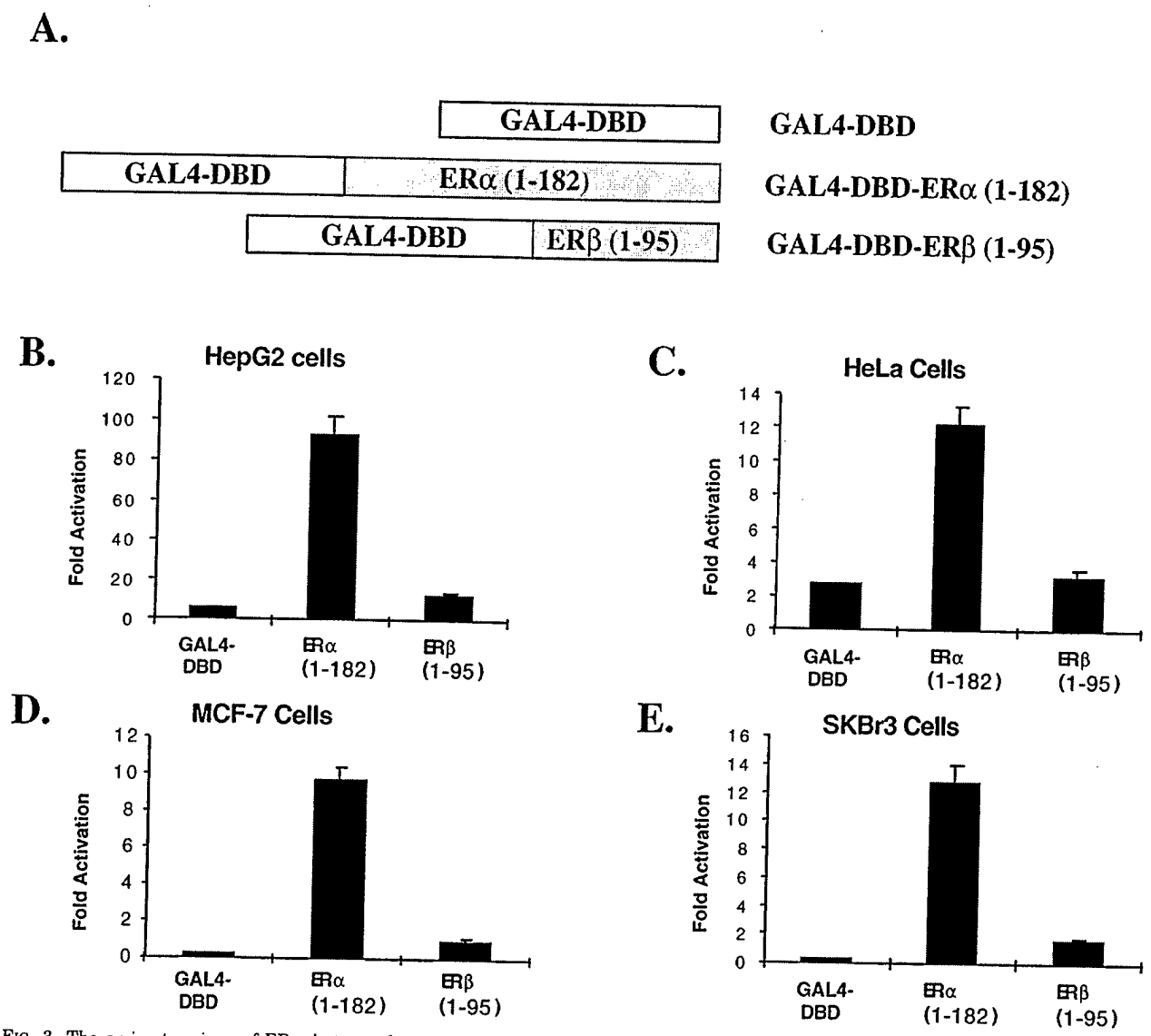


FIG. 3. The amino-terminus of ERα, but not that of ERβ, functions as a strong autonomous activating sequence. A, The GAL4-DBD-ER constructs were created by inserting the amino-terminus of each receptor (amino acids 1–182 of ERα and 1–95 of ERβ) downstream of the GAL4 transcription factor DBD. B–E, HepG2 cells, HeLa cells, MCF-7 cells, and SKBR3 cells were transiently transfected with the GAL4-DBD or GAL4-ER constructs together with a 5x-GAL4-TATA-Luc reporter (containing five copies of the 17-bp palindromic GAL4 transcription factor response element). Cells were harvested after 48 h and assayed for luciferase activity. All transfections were normalized for efficiency using the internal pCMV-β-gal control plasmid. The data are presented as fold activation, where 1 represents a measure of the activity of the GAL4-DBD construct alone. Each *data point* is the average of triplicate determinations.

the system had no effect on ERα transcriptional activity under the conditions of the assay. However, in the presence of 100 pM 17β-estradiol, the activity of ERα was suppressed by the addition of increasing amounts of the ERβ expression vector. At equivalent input vector concentrations, the estradiol-stimulated activity of the coexpressed receptors was repressed to 25% of the activity of ERα alone. These data strongly support the hypothesis that the pharmacology of estrogen(s) will differ in target cells depending on the relative expression levels of ERα and ERβ.

We next compared the activities of ERα, ERβ, or both receptors together over a full range of estradiol concentrations (Fig. 4B). Based on the observation that ERβ functions as a repressor of ERα transcriptional activity at low concentrations of hormone, we predicted that the impact of ERβ on ERα would differ at specific hormone concentrations and that the cellular responsiveness to estradiol would be affected by ERβ expression. Interestingly, we observed that the potency of estradiol in our ERα-dependent transcription systems was right shifted by 1 log when ERβ was coexpressed in the system, whereas the efficacy was unaffected. Similar results were also observed when this experiment was re-

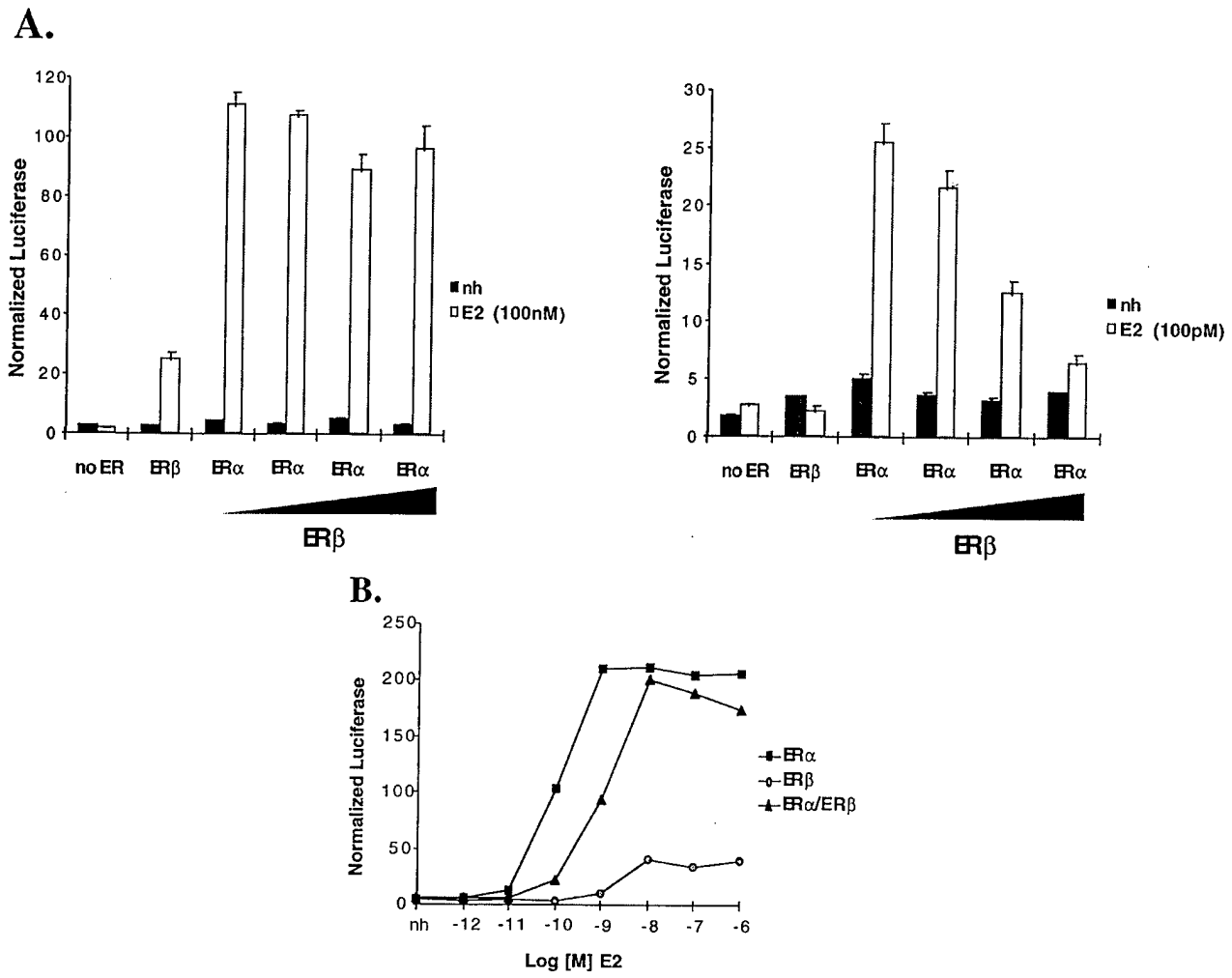


FIG. 4. ERβ is a transdominant repressor of ERα transcriptional activity at subsaturating concentrations of 17β-estradiol. A, HepG2 cells were transiently transfected with the 3x-ERE-TATA-Luc reporter alone (no ER), the reporter and 250 ng ERβ, or the reporter and 250 ng of the ERα expression vector together with increasing concentrations of the ERβ expression vector (0, 10, 50, and 250 ng). After transfection, cells were treated with vehicle (nh) or 100 nM or 100 pM 17β-estradiol (E2). After 48 h, cells were harvested and assayed for luciferase activity, and all transfections were normalized for efficiency using the internal pCMV-β-gal control plasmid. Each *data point* is the average of triplicate determinations, and the average coefficient of variance for each value is less than 10%. B, HepG2 cells were transiently transfected with either ERα or ERβ expression vectors or equal quantities of both vectors together with the C3-Luc reporter and the pCMV-β-gal control plasmid. Cells were induced with vehicle (nh) or increasing concentrations (ranging from 1 pM to 1 μM) of 17β-estradiol (E2) for 48 h, and luciferase assays were performed. Each value was normalized to the β-galactosidase activity. Each *data point* is the average of triplicate determinations, and the average coefficient of variance for each value is less than 10%.

peated in different cellular contexts (data not shown). From these studies, we conclude that 1) ERβ is a transdominant repressor of ERα transcriptional activity at subsaturating concentrations of estradiol; and 2) ERβ expression decreases the sensitivity of ERα-expressing cells to estradiol.

Coexpression of ERβ suppresses the partial agonist activity of tamoxifen through ERα

Our observations that the cellular response to estrogen is dependent on the relative expression of ERα and ERβ suggested that the agonist/antagonist activities of mixed agonists such as tamoxifen may be influenced by the relative expression of the two receptors. To determine the impact of coexpression of the two receptor subtypes on tamoxifen pharmacology, the ERα and ERβ expression vectors were transiently transfected into HepG2 cells, either alone or together, and the cellular response to increasing concentrations of 4-hydroxytamoxifen was measured on the C3-Luc reporter (Fig. 5). As seen before, tamoxifen functioned as an agonist when ERα alone was expressed in the cell and showed no agonist activity when ERβ alone was expressed. In the presence of ERβ, however, the ability of tamoxifen to activate ERα-mediated transcription was completely suppressed. We noticed that the basal transcription in the pres-

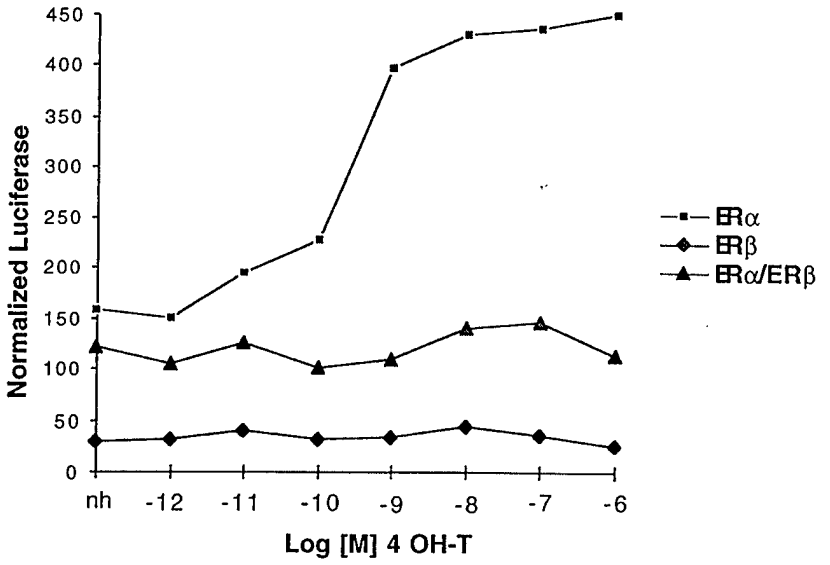


FIG. 5. Coexpression of ERβ suppresses the partial agonist activity of tamoxifen through ERα. HepG2 cells were transiently transfected with either the ERα or ERβ expression vector or equal quantities of both vectors together with the C3-Luc reporter and the pCMV-β-gal control plasmid. Cells were induced with vehicle (nh) or increasing concentrations (ranging from 1 pM to 1 μM) of 4-hydroxytamoxifen (4OH-T) for 48 h, and luciferase assays were performed. Each value was normalized to the β-galactosidase activity. Each *data point* is the average of triplicate determinations, and the average coefficient of variance for each value is less than 10%.

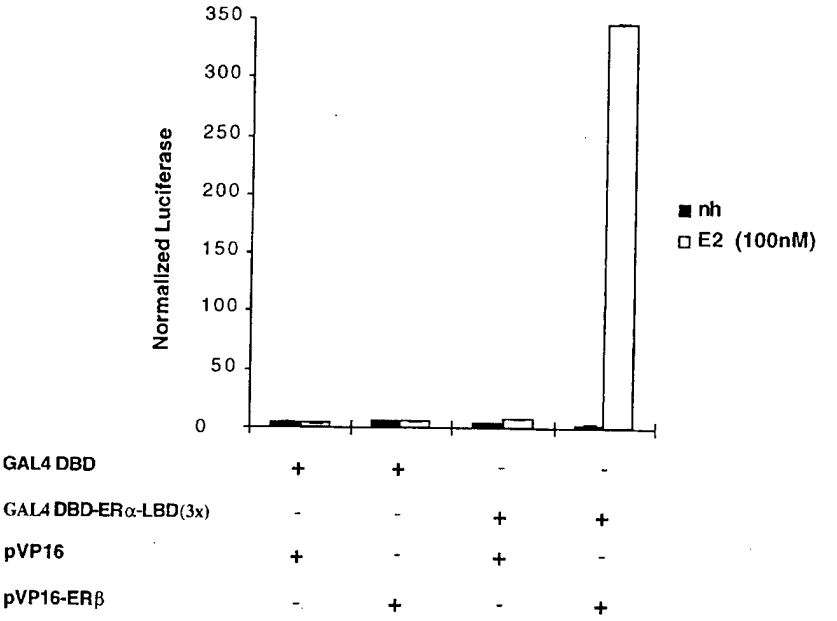


FIG. 6. ERα and ERβ form heterodimers *in vivo*. HepG2 cells were transiently transfected with either GAL4-DBD or GAL4-ERα-LBD(3X) (in this construct AF-2 activity is removed to decrease the basal activity, but the dimerization domain is intact) together with either pVP16 or pVP16-ERβ and the 5x-GAL4-TATA-Luc reporter. Cells were treated with vehicle (nh) or 100 nM 17β-estradiol (E2) for 48 h, and luciferase assays were performed. Each luciferase value was normalized to the β-galactosidase activity. Each *data point* is the average of triplicate determinations, and the average coefficient of variance for each value is less than 10%.

ence of both receptors was intermediate between ERα and ERβ alone in this assay. Although we have not pursued this interesting finding further, we do know that all of the basal activity observed in the presence of ERα is ER-dependent. Thus, ERβ may effectively inhibit tamoxifen-dependent ERα-mediated transcription, but not that activity mediated through the same receptor in the absence of ligand. Overall, these results suggest that the tissue-selective agonist activity of antiestrogens may be determined by the relative expression of ERα and ERβ in a tissue.

ERα and ERβ form heterodimers in vivo

We next investigated the molecular mechanism by which ERβ modulates ERα transcriptional activity. It has recently been reported that coexpression of ERα and ERβ results in the preferential formation of receptor heterodimers (instead of homodimers) that are capable of activating transcription from estrogen-responsive enhancers (20, 21). Our observation that classical ERα- and ERβ-mediated responses are modified when the two receptors are coexpressed suggests that these alternative responses could be mediated through receptor heterodimers that may possess unique transcriptional activities. To address this issue we examined the interaction between the two receptors in a mammalian two-hybrid assay. HepG2 cells were transfected with the pBKC-DBD-ERα-LBD(3x) vector and pVP16-ERβ vectors together with a GAL4-responsive reporter and treated with vehicle or 100 nM 17β-estradiol (Fig. 6). The ERα-LBD(3x) vector con-

tains three point mutations in the AF-2 domain that abolish transcriptional activity but have no effect on ER dimerization (11). We observed that ERα and ERβ show a strong hormone-dependent interaction. Furthermore, our results show that ERα and ERβ heterodimerize *in vivo* and support the recent findings that heterodimers are formed when the two subtypes are coexpressed. Although previous studies have shown that ERα and ERβ heterodimerize in the absence of hormone (20, 21), these studies were carried out *in vitro* and may not accurately represent what occurs in the cell where ERα and ERβ do not exist in isolation but, rather, interact with a variety of proteins. Overall, the results imply that the impact of each receptor on the other's pharmacology may be mediated at least in part through the formation of heterodimers with properties that may be different from those of the homodimers.

ERβ interacts with target gene promoters in the absence of ligand

We have observed that ERβ suppresses ERα-mediated transcription at subsaturating levels of hormone. One possible explanation for this activity is that ERβ may bind its cognate response element in a constitutive manner and thus compete with ERα for access to the DNA target. To test this hypothesis, ERα and ERβ were fused to the VP16 activation domain in order to bypass the need for ligand to activate the AFs within the two receptors. Thus, when tethered to DNA, the chimeras will activate transcription regardless of the nature of the bound ligand. HepG2 cells were transiently transfected with increasing concentrations of the pVP16-ERα or pVP16-ERβ expression plasmids together with the 3x-ERE-TATA-Luc reporter in the presence of vehicle or 100 nM 17β-estradiol. As shown in Fig. 7A, when overexpressed, VP16-ERα did have the capacity to interact with DNA in the absence of hormone. However, when VP16-ERα was limiting, hormone had a pronounced effect on DNA binding. To eliminate the possibility that the elevated transcriptional responses of VP16-ERα in the presence of agonist were due to synergy between the ERα activation functions and the VP16 activation domain in the presence of estradiol, we repeated the experiments using VP16-ERα(3x), which contains three point mutations in the ERα AF-2 domain that disrupt the ligand-dependent coactivator binding pocket and nullify the transcriptional activity of the receptor. Under these conditions we observed that hormone was required for DNA binding, even at high receptor concentrations (Fig. 7B). In contrast to the results obtained with VP16-ERα, we observed that the ability of VP16-ERβ to interact with DNA was entirely ligand independent, regardless of the level of expression (Fig. 7C). These studies reveal another mechanistic difference between ERα and ERβ and suggest that ERβ regulates ERα-mediated transcriptional activity at the level of DNA-binding site competition.

Discussion

Differential activities of nuclear hormone receptor subtypes

The ability of two nuclear receptor subtypes to display distinct or even opposing transcriptional activities is an emerging paradigm in nuclear receptor signaling. One example is the human glucocorticoid receptor (GR), which exists in two forms (α and β) that arise by an alternate splicing event that results in a truncation of the C-terminus in the GRβ form (22). Analysis of the transcriptional properties of these two receptors indicated that they both recognize the same intracellular targets, but GRβ is unable to bind agonists (22). Therefore, although GRα can enhance the transcription of glucocorticoid-responsive enhancers, GRβ is not transcriptionally active and can act as a dominant negative inhibitor of GRα activity. The contrasting activities of the GRs suggest that the relative tissue distributions of the two receptors are a major determinant of the biological effects of glucocorticoid compounds. The existence of two forms of the human PR, each with different biological activity, extends the concept that receptor isoforms are an important part of steroid hormone receptor pharmacology. The two PRs are identical in sequence, with the exception that hPR-B has an additional 164 amino acids within its N-terminus originating from an alternate translation initiation event. These isoforms have been shown to bind the same ligands and regulate transcription of the same genes. However, in most cell types PR-B is a transcriptional activator, whereas PR-A acts as a transdominant repressor of PR-B. Interestingly, in the presence of an activating ligand, human PR-A can inhibit the transcriptional activities of ER, GR, androgen receptor, and mineralocorticoid receptor (15).

Our studies suggest that the human ERα and ERβ provide yet another example of two nuclear receptor subtypes that demonstrate distinct transcriptional activities. As is seen with human PRs, the differential activities of the two ERs arise from functional variations in the receptor N-termini. ERα contains a constitutive AF-1 in the N-terminus that functions in a cell- and promoter-specific manner to enhance the overall transcriptional response of the receptor. However, the corresponding region of ERβ lacks significant transcriptional activity and contains a repressor domain that decreases the overall transcriptional activity of the receptor. This inhibitory region functions only in the context of the intact receptor, as has been shown for the inhibitory N-terminus of PR-A (15) and for the repressor domains of the transcription factor c-Fos (23). Our studies do not eliminate the possibility that ERβ does, in fact, have an AF-1, but suggest that its function is masked by the presence of an amino-terminal repressor domain. This hypothesis is supported by the fact that the N-terminus of ERβ showed low levels of autonomous transcriptional activity when fused to the heterologous GAL4-DBD. Furthermore, recent studies have shown that ERβ transcriptional activity can be stimulated by the mitogen-activated protein kinase pathway (23a), and that this activity appears to require an intact ERβ amino-terminus. Mutational analysis will be required to determine whether ERβ contains separate activation and repression domains within its amino-terminus.

An additionally important distinction between ERα and ERβ resulted from our finding that ERα-AF-1 and AF-2 act synergistically under most circumstances, whereas the AF-2 of ERβ functions as an independent activation domain. It is likely, therefore, that ERα and ERβ will display differences in their preferences for coactivators and corepressors in target cells. In recent years, several transcriptional coactivator

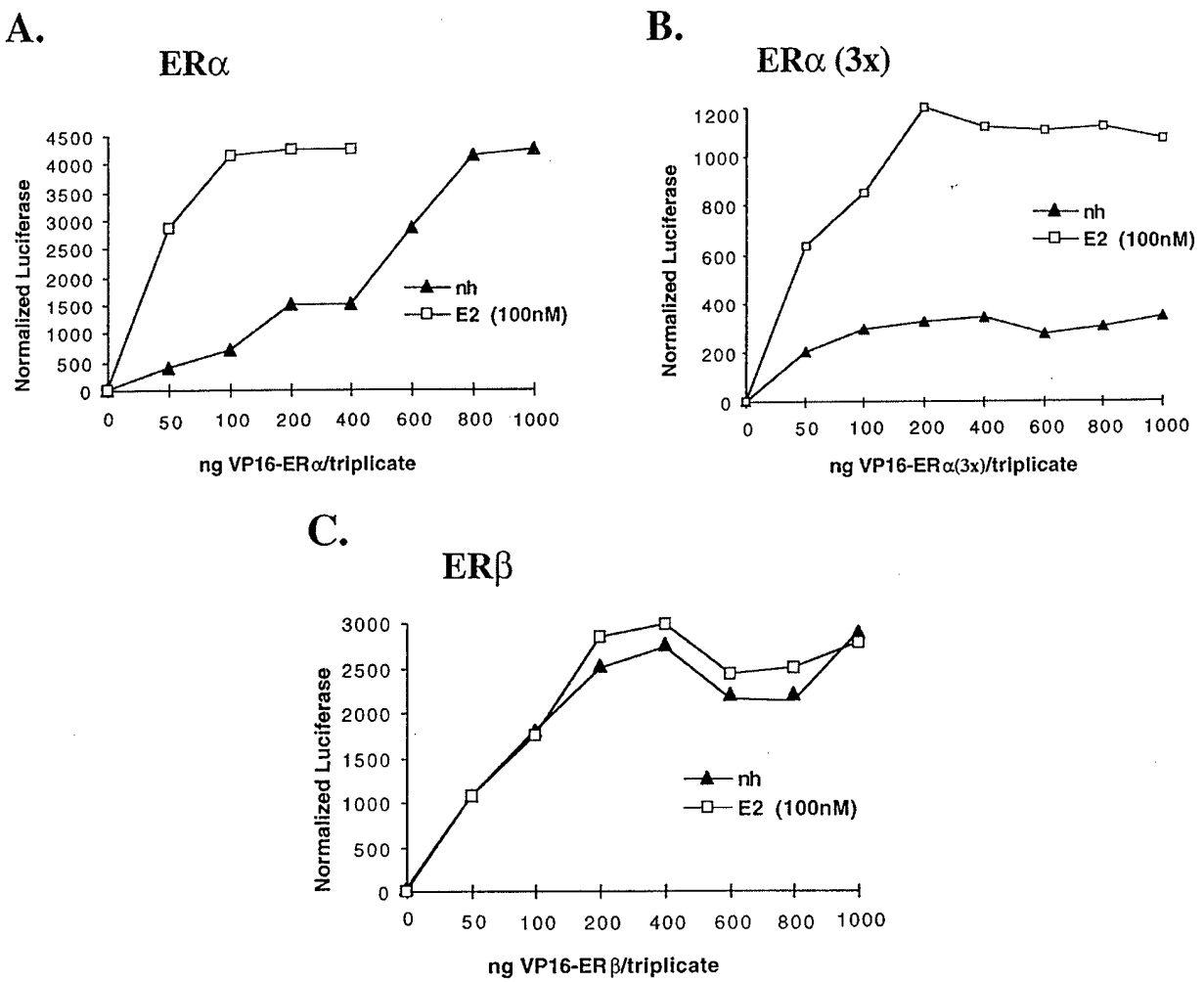


FIG. 7. ERβ interacts with target gene promoters in the absence of ligand. HepG2 cells were transiently transfected with increasing concentrations of the pVP16-ERα expression vector (A), the pVP16-ERα(3x) expression vector (B), or the pVP16-ERβ expression vector (C). Each expression construct includes the VP16 activation domain sequence fused 5′ to the entire coding sequence for the human ERα, ERα(3x) mutant, or ERβ. After transfection, cells were treated with vehicle (nh) or 100 nM 17β-estradiol (E2) for 48 h, and luciferase assays were performed. Each value was normalized to the β-galactosidase activity. Each *data point* is the average of triplicate determinations, and the average coefficient of variance for each value is less than 10%.

proteins have been identified that interact with the hormone-binding domain of nuclear receptors and are thought to mediate the activity of AF-2. Specifically, the coactivators steroid receptor coactivator-1 (SRC-D), amplified in breast cancer (AIB1), transcriptional intermediary factor-2 (TIF-2), and glucocorticoid receptor interacting protein (GRIP-1) (murine homolog of TIF-2) and the cointegrator CBP/p300 have been shown to potentiate the activity of ERα and other nuclear receptors (24–27). The C-termini of both ERα and ERβ have been shown to bind the coactivators SRC-1 and GRIP-1 in an agonist-dependent manner (28, 29) (our unpublished results). Therefore, it is possible that although the cofactors that mediate transcriptional activity of the two ERs are the same, these proteins may interact with distinct regions of each receptor or differentially recruit other cellular factors when bound to one receptor *vs.* the other. Although sequence comparisons suggest that the N-terminus may be the primary discriminator by which coactivators can interact with the receptors, the fact that AF-2 of ERα and ERβ are also functionally different suggests that the process is much more complex. It is not clear at this time whether AF-1 and/or AF-2 interact with distinct coactivators or if these domains have independent contact sites on the same coactivator. Regardless, it is clear that in the context of ERα, AF-1 is required for maximal agonist-induced transcriptional activity, as mutations in this domain have been shown to abolish tamoxifen partial agonist activity and dampen the response to estrogen (30). The absence of an efficient AF-1 in ERβ clearly influences the manner in which coactivators interface with this receptor and ultimately its ligand responsiveness. Our data are also compatible with the concept that the N-terminus of ERβ binds a protein that has an autonomous inhibitory ac-

tivity or one that inhibits transcriptional activity by blocking the binding of coactivators to AF-1 and AF-2. If this is the case, then it is possible that in tissues in which the putative repressor protein is absent, antiestrogens could manifest partial agonist activity, and agonists would be more potent receptor activators.

Roles of ERα and ERβ in determining cellular sensitivity to estrogen

One of the most important findings of this study is that the relative levels of ERα and ERβ are an important determinant of cellular sensitivity to estrogens. Although ERα is the stronger transcriptional activator of the two ER isoforms, at physiological concentrations of estradiol, coexpression of ERβ results in suppression of both the efficacy and the potency of hormone-stimulated responses. This suggests that it will be important to determine the extent to which the two receptors colocalize in order to more accurately predict the biological responses to ER agonists in specific target tissues. The ability of ERβ to function as a transcriptional inhibitor or activator, depending on the agonist concentration, suggests that completely different patterns of gene expression may be observed at different hormone levels. In addition, the ability of ERβ to switch from a transcriptional repressor to an activator as estradiol levels rise may provide cells expressing both isoforms with a mechanism to control cellular sensitivity to hormones. Such a process could explain why during the early part of the menstrual cycle, low plasma concentrations of estradiol exhibit an inhibitory effect on gonadotropin secretion, whereas when levels of hormone are elevated during the late follicular phase, the pituitary release of LH and GnRH secretion from the hypothalamus is enhanced. In light of the recent localization of ERβ to rat hypothalamic neurons projecting to the pituitary (31, 32), it is possible that the balance between ERα and ERβ activities in these tissues may mediate the differential sensitivities to estrogens throughout the menstrual cycle. The role of ERβ in the regulation of cellular responsiveness to agonists may merit consideration in dosing regimens of estrogen-like pharmaceutical compounds, as it is likely that fluctuations in the bioavailability of receptor activating ligands may have a greater impact in tissues where ERα and ERβ colocalize. This may be particularly important in ER-positive breast tumors, where it has been shown that ERβ, in addition to ERα, may be expressed (33).

Our studies also suggest that the relative levels of ERα and ERβ are an important determinant of the pharmacology of antiestrogens. The observation that tamoxifen is a more potent competitive antagonist of ERβ (8) and does not display agonist activity on the receptor raises the possibility that there will be a better response to tamoxifen in ERβ-positive tumors. In view of our finding that ERβ suppresses the partial agonist activity of tamoxifen on ERα, it will be interesting to determine whether tumors expressing both subtypes show a better response to tamoxifen as well. It will also be important to determine whether ERβ is down-regulated in tamoxifen-resistant tumors as an adaptive mechanism for growth.

A working model to explain the cross-talk between ERα and ERβ

We have developed a working model to explain how ERβ can regulate ERα transcriptional activity in cells where the receptors are coexpressed. This model is based on two fundamental observations: 1) ERβ binds to target gene promoters in a ligand-independent manner; and 2) ERβ can form heterodimers with ERα within cells. Thus, in the presence of low subsaturating concentrations of hormone, inactive ERβ binds to its target response element and competitively blocks ERα binding. As hormone levels rise, the amount of activated ERα and ERβ also rises, sufficient activated receptor is formed to compete with the unliganded, inactive ERβ, and transcription can proceed. A purely competitive interaction would predict that as ERβ levels rise, agonist efficacy would decrease to a level approaching that observed when ERβ alone is expressed in cells. However, the observation that under conditions of hormone excess, overexpression of ERβ does not decrease the efficacy of estradiol suggests that the interaction of ERα and ERβ is more complex. We believe that under hormone-saturating conditions, ERα and ERβ can form heterodimers and that the transcriptional activity of the heterodimer is indistinguishable from that of the ERα homodimer. Although difficult to address experimentally, it is possible that of the three potential ER complexes, the ERβ homodimer has the highest affinity for corepressors and/or the lowest affinity for coactivators, and consequently, it is the least transcriptionally active. However, in the context of a heterodimer, the presence of ERα assists ERβ in recruiting cofactors such as SRC-1 and GRIP-1. Thus, the resultant complex of ERα/ERβ and their associated coactivators is indistinguishable from that formed by an ERα homodimer. If this latter model is found to be true, then we would predict that the major role of ERβ is to modulate ERα transcriptional activity at low hormone levels.

Acknowledgments

We thank Drs. Mark Nuttall and X. F. Wang for providing plasmids. We thank T. Willson for providing reagents and for sharing unpublished data.

References

1. **Evans RM** 1988 The steroid and thyroid receptor superfamily. Science 240:889–895
2. **Tsai M, O'Malley BW** 1994 Molecular mechanisms of action of steroid/thyroid receptor superfamily members. Annu Rev Biochem 63:451–486
3. **Aronica SM, Katzenellenbogen BS** 1993 Stimulation of estrogen receptor-mediated transcription and alteration in the phosphorylation state of the rat uterine estrogen receptor by estrogen, cyclic adenosine monophosphate, and insulin-like growth factor-I. Mol Endocrinol 7:743–752
4. **Ignar-Trowbridge DM, Teng CT, Ross KA, Parker MG, Korach KS, McLachlan JA** 1993 Peptide growth factors elicit estrogen receptor-dependent transcriptional activation at an estrogen-responsive element. Mol Endocrinol 7:992–998
5. **Curtis SW, Washburn T, Sewall C, DiAugustine R, Lindzey J, Couse JF, Korach KS** 1996 Physiological coupling of growth factor and steroid receptor signaling pathways: estrogen receptor knockout mice lack estrogen-like responses to epidermal growth factor. Proc Natl Acad Sci USA 93:12626–12630
6. **Kuiper GGJM, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson J-A** 1996 Cloning of a novel estrogen receptor expressed in rat prostate and ovary. Proc Natl Acad Sci USA 93:5925–5930
7. **Mosselman S, Polman J, Dijkema R** 1996 ERβ: identification and characterization of a novel human estrogen receptor. FEBS Lett 392:49–53
8. **Kuiper GGJM, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S, Gustafsson J-A** 1997 Comparison of the ligand binding specificity and tran-

script tissue distribution of estrogen receptors α and β. Endocrinology 138:863–870
9. **Couse JF, Lindsey J, Grandien K, Gustafsson J-A, Korach KS** 1997 Tissue distribution and quantitative analysis of estrogen receptor-α (ERα) and estrogen receptor-β (ERβ) messenger ribonucleic acid in the wild-type and ER-α knockout mouse. Endocrinology 138:4613–4621
10. **Berry M, Metzger D, Chambon P** 1990 Role of the activating domains of the oestrogen receptor in the cell-type and promoter-context dependent agonistic activity of the anti-oestrogen 4-hydroxytamoxifen. EMBO J 9:2811–2818
11. **Tzuckerman MT, Esty A, Santiso-Mere D, Danielian P, Parker MG, Stein RB, Pike JW, McDonnell DP** 1994 Human estrogen receptor transactivational capacity is determined by both cellular and promoter context and mediated by two functionally distinct intramolecular regions. Mol Endocrinol 8:21–30
12. **Willson TM, Norris JD, Wagner BL, Asplin I, Baer P, Brown HR, Jones SA, Henke B, Sauls H, Wolfe S, Morris DC, McDonnell, DP** 1997 Dissection of the molecular mechanism of action of GW5638, a novel estrogen receptor ligand, provides insights into the role of estrogen receptor in bone. Endocrinology 138:3901–3911
13. **McDonnell DP, Dana SL, Hoener PA, Lieberman BA, Imhof MO, Stein RB** 1995 Cellular mechanisms which distinguish between hormone- and antihormone-activated estrogen receptor. Ann NY Acad Sci 761:121–137
14. **Vegeto E, Shahbaz MM, Wen, DX, Goldman ME, O'Malley BW, McDonnell DM** 1993 Human progesterone receptor A form is a cell- and promoter-specific repressor of human progesterone receptor B function. Mol Endocrinol 7:1244–1255
15. **Giangrande PH, Pollio G, McDonnell DP** 1997 Mapping and characterization of the functional domains responsible for the differential activity of the A and B isoforms of the human progesterone receptor. J Biol Chem 52:32889–32900
16. **Norris JD, Fan D, Wagner BL, McDonnell DP** 1996 Identification of the sequences within the human C3 promoter required for estrogen responsiveness provides insight into the mechanism of tamoxifen mixed agonist activity. Mol Endocrinol 10:1605–1616
17. **Norris JD, Fan D, Aleman C, Marks JR, Futreal PA, Wiseman RW, Iglehart JD, Deininger PL, McDonnell DP** 1995 Identification of a new subclass of Alu DNA repeats which can function as estrogen receptor-dependent transcriptional enhancers. J Biol Chem 270:22777–22782
18. **Schreiber E, Matthias P, Muller MM, Schaffner, W** 1998 Identification of a novel lymphoid specific octamer binding protein (OTF-2B) by proteolytic clipping bandshift assay (PCBA). EMBO J 7:4221–4229
19. **Moore JT, McKee DD, Moore LB, Jones SA, Su J-L, Slentz-Kesler K, Horne EL, Kliewer SA, Lehmann JM, Willson TM** 1998 Cloning, ligand binding and functional activity of human estrogen receptor β isoforms. Biochem Biophys Res Commun 247:75–78
20. **Cowley SM, Hoarse S, Mosselman S, Parker MG** 1997 Estrogen receptors α and β form heterodimers on DNA. J Biol Chem 272:19858–19862
21. **Pettersson K, Grandien K, Kuiper GGJM, Gustafsson J-A** 1997 Mouse estrogen receptor β forms estrogen response element-binding heterodimers with estrogen receptor α. Mol Endocrinol 11:1486–1496
22. **Oakley RH, Sar M, Cidlowski JA** 1996 The human glucocorticoid receptor β isoform. Expression, biochemical properties, and putative function. J Biol Chem 271:9550–9559
23. **Brown HJ, Sutherland JA, Cook A, Bannister AJ, Kouzarides T** 1995 An inhibitor domain in c-Fos regulates activation domains containing the HOB1 motif. EMBO J 14:124–131

23a. **Tremblay A, Tremblay GB, Labrie F, Giguere V** 1999 Ligand-independent recruitment of SRC-1 to estrogen receptor beta through phosphorylation of activation function AF-1. Mol Cell 3:513–519

24. **Horwitz KB, Jackson TA, Bain DL, Richer JK, Takimoto GS, Tung L** 1996 Nuclear receptor coactivators and corepressors. Mol Endocrinol 10:1167–1177
25. **Onate SA, Tsai S-Y, Tsai M-J, O'Malley BW** 1995 Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. Science 270:1354–1357
26. **Eng FCS, Barsalou A, Akutsu N, Mercier I, Zechel C, Mader S, White JH** 1998 Different classes of coactivators recognize distinct but overlapping binding sites on the estrogen receptor ligand binding domain. J Biol Chem 273:28371–24377
27. **Shibata H, Spencer TE, Onate SA, Jenster G, Tsai S-Y, Tsai M-J, O'Malley BW** 1997 Role of co-activators and co-repressors in the mechanism of steroid/thyroid receptor action. Recent Prog Horm Res 52:141–164
28. **Hong H, Kohli K, Trivedi A, Johnson DL, Stallcup MR** 1996 GRIP1, a novel mouse protein that serves as a transcriptional coactivator in yeast for the hormone binding domains of steroid receptors. Proc Natl Acad Sci USA 93:4948–4952
29. **Tremblay GB, Tremblay A, Copeland NG, Gilbert DJ, Jenkins NA, Labrie F, Giguere V** 1997 Cloning, chromosomal localization, and functional analysis of the murine estrogen receptor β. Mol Endocrinol 11:353–365
30. **McInerney EM, Katzenellenbogen BS** 1996 Different regions in activation function-1 of the human estrogen receptor required for antiestrogen- and estradiol-dependent transcription activation. J Biol Chem 271:24172–24178
31. **Alves SE, Lopez V, McEwen BS, Weiland NG** 1998 Differential colocalization of estrogen receptor β (ERβ) with oxytocin and vasopressin in the paraventricular and supraoptic nuclei of the female rat brain: an immunocytochemical study. Proc Natl Acad Sci USA 95:3281–3286
32. **Shughrue PJ, Komm B, Merchenthaler I** 1996 The distribution of estrogen receptor-β mRNA in the rat hypothalamus. Steroids 61:678–681
33. **Dotzlaw H, Leygue E, Watson PH, Murphy LC** 1996 Expression of estrogen receptor-β in human breast tumors. J Clin Endocrinol Metab 82:2371–2374

Development of Peptide Antagonists That Target Estrogen Receptor β-Coactivator Interactions

Julie M. Hall, Ching-yi Chang, and Donald P. McDonnell

Department of Pharmacology and Cancer Biology
Duke University Medical Center
Durham, North Carolina 27710

The biological actions of estrogen are manifest through two genetically distinct estrogen receptors (ERα and ERβ) that display nonidentical expression patterns in target tissues. The phenotypic alterations in response to estrogens in mice disrupted for either or both of these receptors are not identical, suggesting that each subtype plays a unique role in ER-action. However, the lack of subtype-specific agonists and antagonists has made it difficult to define the processes that are regulated by ERα and/or ERβ. Previously, we have reported the identification and characterization of a series of LXXLL-containing peptide antagonists that block estrogen signaling by preventing the association of ERα with required coactivators. As expected, given the similarity of the coactivator binding pockets among nuclear receptors, most of the peptide antagonists identified inhibited the activity of multiple receptors. However, by altering sequences flanking the core LXXLL motif, some receptor selectivity was afforded. Building on this observation, we have screened combinatorial phage libraries, expressing peptides in the format X_7LXXLLX_7, for peptides that interact in a specific manner with ERβ. Using this approach, a series of highly specific, potent peptide antagonists have been identified that efficiently inhibit ERβ-mediated estrogen signaling when introduced into target cells. Interestingly, in cells where both ER subtypes were expressed, these ERβ antagonists were capable of attenuating ER action, suggesting that ERα and ERβ do indeed form functional heterodimeric complexes. We believe that suitably formulated versions of these peptides can be used to study ERβ action *in vitro* and *in vivo*. In addition, the unanticipated specificity of the peptides identified should serve as an impetus to investigate the use of this approach to develop peptide antagonists of other nuclear receptors and unrelated transcription factors. (Molecular Endocrinology 14: 0000–0000, 2000)

0888-8809/00/$3.00/0
Molecular Endocrinology 14(12): ●●●–●●●

INTRODUCTION

The human estrogen receptor (ER) belongs to the nuclear receptor superfamily of ligand-inducible transcription factors (1), whose members include the receptors for steroids, thyroid hormone, retinoic acid, vitamin D, and orphan receptors for which no ligands have yet been identified. The mechanism of action of ER is similar to that of other nuclear receptors (2). In the absence of hormone, the receptor is sequestered within the nuclei of target cells in an inactive state. The binding of ligand induces an activating conformational change within ER, an event that permits the receptor to interact with transcriptional coactivators such as steroid receptor coactivator 1 (SRC-1) and glucocorticoid receptor interacting protein 1 (GRIP1) (3, 4), and which facilitates the association of the resulting complex with specific DNA response elements (EREs) located within the regulatory regions of target genes. Depending on the promoter context, the DNA-bound receptor can then exert either a positive or negative effect on target gene transcription (2, 5).

Until recently it was thought that all of the biological actions of estrogens and antiestrogens were manifest through a single receptor located within target cell nuclei. However, the identification of a second estrogen receptor, ERβ (6, 7), has indicated that estrogen signaling is more complex. The two ER subtypes, ERα and ERβ, share extensive amino acid similarity in their ligand- and DNA-binding domains, but minimal homology within their amino-terminal regions. Not surprisingly therefore, these receptors exhibit similar, but not identical, ligand binding characteristics (8) and interact with the same DNA response elements. The most obvious difference between the two receptors is that ERα is a more efficient activator of ERE-containing genes than ERβ under most circumstances (7, 9–11). In addition, it has been noted that ERβ can interact in a constitutive manner with target promoters

and can attenuate the ligand-activated transcriptional activity of ERα (11). Thus, in cells where both receptors are expressed, overall estrogen responsiveness is reduced.

In parallel with studies performed *in vitro*, the creation and characterization of mice in which either ERα and/or ERβ have been disrupted (αERKO, βERKO and αβERKO, respectively) have demonstrated that the two receptors are not functionally equivalent and that each subtype plays a unique role in ER action *in vivo* (12, 13). However, it is clear that in addition to these mouse models, there is a need for subtype-selective agonists and antagonists that will permit the transient manipulation of receptor function in intact animals. Consequently, to complement the efforts of others who are engaged in screening for small molecules that interact with the ligand-binding pockets of ERα and ERβ (15), we have undertaken a novel approach to develop subtype-specific antagonists that inhibit ERβ action in a manner distinct from known antiestrogens.

All of the currently available ER antagonists function by 1) binding to the receptor ligand-binding domain, thereby blocking agonist access, and 2) inducing a conformational change within the receptor that prevents it from interacting efficiently with transcriptional coactivators such as SRC-1, GRIP1, and amplified in breast cancer 1 (AIB-1). Specifically, it has been shown that agonist binding to the receptor induces a conformational change that permits the formation of a hydrophobic pocket (16, 17), enabling the receptor to interact with the LXXLL motif contained within the receptor interaction domains of most of the validated coactivators (18, 19). The conformational changes induced in ER upon antagonist binding do not permit coactivator recruitment (19). Clearly, the most direct method of inhibiting ER function would be to develop drugs that bind directly to the coactivator binding pockets within ERα or ERβ and block coactivator recruitment. Given that the coactivator binding pockets in ERα, ERβ, and other nuclear receptors are structurally similar and that most of the known coactivators do not appear to demonstrate receptor selectivity, it was not obvious that the receptor-cofactor binding pocket was a bona fide drug target. However, we have recently identified a series of LXXLL-containing peptides that interact very well with the coactivator binding pocket of ERβ, but which demonstrate distinct preferences in their ability to interact with other receptors (20). Thus, all LXXLL motifs are not functionally equivalent. Building on this observation, in the current study we have identified LXXLL-containing peptides that interact specifically with ERβ and inhibit its transcriptional activity. We believe that these novel peptide antagonists will serve as useful tools to evaluate the role of ERβ in estrogen signaling. In addition, we anticipate that the general approach used to obtain these ERβ antagonists can be applied to the development of peptide antagonists of other nuclear receptors and unrelated transcription factors.

RESULTS

Affinity Selection of ERβ-Binding, LXXLL-Containing Peptides Using Phage Display

A critical step in ER action is the ligand-dependent recruitment of transcriptional coactivators to target gene promoters. Antiestrogens manifest their inhibitory activities by altering ER structure and independently blocking cofactor binding (19, 22). In this study, the feasibility of targeting ER-coactivator interactions directly as a mechanism of developing human ERβ-specific antagonists was evaluated. Using combinatorial phage display technology (21), we created and screened a phage library that expressed 19-mer peptides containing a central fixed Leu-X-X-Leu-Leu motif flanked by seven random amino acids on each side. Since we used an NNK nucleic acid format in the construction of this library it has a theoretical complexity of 1.2×10^{24}. However, since our library contained only 10^8 independent clones, we are only surveying a fraction of the potential LXXLL motifs that can interact with ERβ. This random LXXLL library was screened for phage that bound to ERβ either in the absence or presence of estradiol, and 70 of the most avid interactors identified were brought forward for further analysis. A secondary enzyme-linked immunosorbent assay (ELISA) was used to confirm the ERβ-binding characteristics of the plaque-purified phage. Cross-screening, using a similar approach, revealed that 37 of the phage identified bound to both ER subtypes whereas 33 interacted selectively with ERβ. The latter subset of phage expressing LXXLL-containing peptides were brought forward for further analysis.

Characterization of the ERβ-Selective LXXLL-Containing Peptides in Intact Cells

We performed a mammalian two-hybrid assay to assess the ability of the peptide sequences identified by phage display to interact selectively with ERβ in intact cells. Each of the 33 ERβ-selective peptides identified *in vitro* were expressed as a yeast Gal 4 DNA-binding domain (Gal4DBD) fusion protein and tested for their ability to interact with ERα and/or ERβ expressed as fusions to the VP16 activation domain. Expression of the peptide-fusions was confirmed by Western immunoblotting (data not shown). A Gal4DBD-fusion of the NR-box of the ER coactivator SRC-1 (containing three LXXLL motifs), shown previously to interact with both ERα and ERβ, was used in this assay as a positive control. When analyzed in the two-hybrid assay, it was found that 15 of the 33 peptides studied interacted with ERβ, but not ERα. Representative examples are shown in Fig. 1 of those peptides that were isolated in the screen with the apo-ERβ (Fig. 1A), and those identified with the agonist-liganded receptor (Fig. 1B). Interestingly, several of the peptides were able to bind ERβ in the absence of ligand, suggesting either that a portion

F1

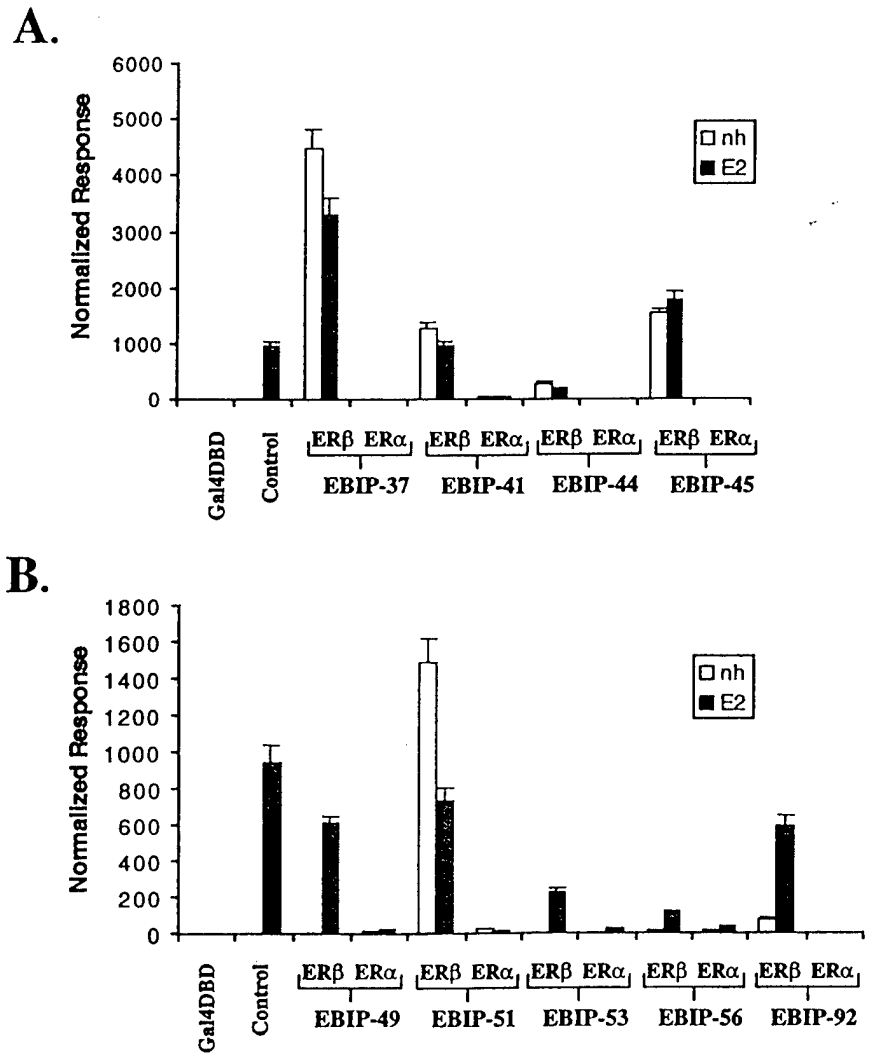


Fig. 1. Evaluation of ERβ-Selective LXXLL-Containing Peptides in Intact Cells

Mammalian two-hybrid assays were performed to examine the interactions between the peptides and ERα or ERβ in mammalian cells. Each ER expression construct includes the VP16 activation domain sequence fused 5′ to the complete coding sequence for the human ERα or ERβ. A, Analysis of the interaction of peptides that were isolated in the screen with apo-ERβ (four representative peptides are shown). B, Analysis of the interaction of peptides that were isolated in the screen with agonist-liganded ERβ (five representative peptides are shown). HepG2 cells were transiently transfected with the pVP16-ERα expression vector or the pVP16-ERβ expression vector together with each peptide-Gal4DBD fusion construct, the 5x-GAL4-TATA-Luc reporter, and the pCMV-β-gal control plasmid. The SRC-1 (NR-box)-Gal4DBD fusion was used as a control. Cells were induced with vehicle (nh) or 10^{-7} M 17β-estradiol, (E_2) for 24 h, and luciferase assays were performed. Each value was normalized to the β-galactosidase activity. Each data point is the average of triplicate determinations, and the average coefficient of variance for each value is <10%.

of the ERβ aporeceptor resides in an active conformation, or that the binding of LXXLL-containing motifs to the receptor may facilitate activation. Using the mammalian-two hybrid assay, it was also shown that the introduction of inactivating point mutations into the AF-2 region of ERβ (11) completely abolished the interaction of each peptide with the receptor (see Fig. 7A, below). These results suggested that the peptides were targeting the coactivator binding pocket, and thus would be able to antagonize ERβ transcriptional activity.

ERβ-Selective LXXLL Peptides Display Hormone Specificity

The interaction of LXXLL-containing coactivators such as SRC-1 or GRIP1 with ERα and ERβ has been shown to require agonist activation of the receptor (3–5). The surprising finding that some LXXLL-containing peptides can interact with apo-ER begged a re-evaluation of the role of ligand in regulating ERβ-LXXLL interactions. This was accomplished using the mammalian two-hybrid assay to examine the effect of

different ER ligands on peptide binding. As expected, the interactions between the Gal4DBD-SRC-1, and Gal4DBD-GRIP1 NR-box fusions and ERβ were enhanced by the addition of the agonists 17β-estradiol and genistein (Fig. 2A). However, administration of antiestrogens alone antagonized the basal receptor-peptide interactions. Interestingly, both of the control peptide fusions showed significant levels of hormone-independent interaction with ERβ. A similar observation was made *in vitro*, where glutathione-S-transferase (GST)-pull down assays revealed that both SRC-1 and GRIP1 interacted with ERβ in the absence of hormone (data not shown).

When the ERβ-selective peptides were analyzed in this assay, we were able to divide them into two classes: those that interacted with the receptor in the absence of hormone (Fig. 2B) and those whose interaction was strictly agonist dependent (Fig. 2C). The profiles of three representative peptides from each group are shown. Several of the peptide fusions ((ERβ-interacting peptides EBIP-37 and EBIP-44) interacted equally well with ERβ in the absence of hormone and

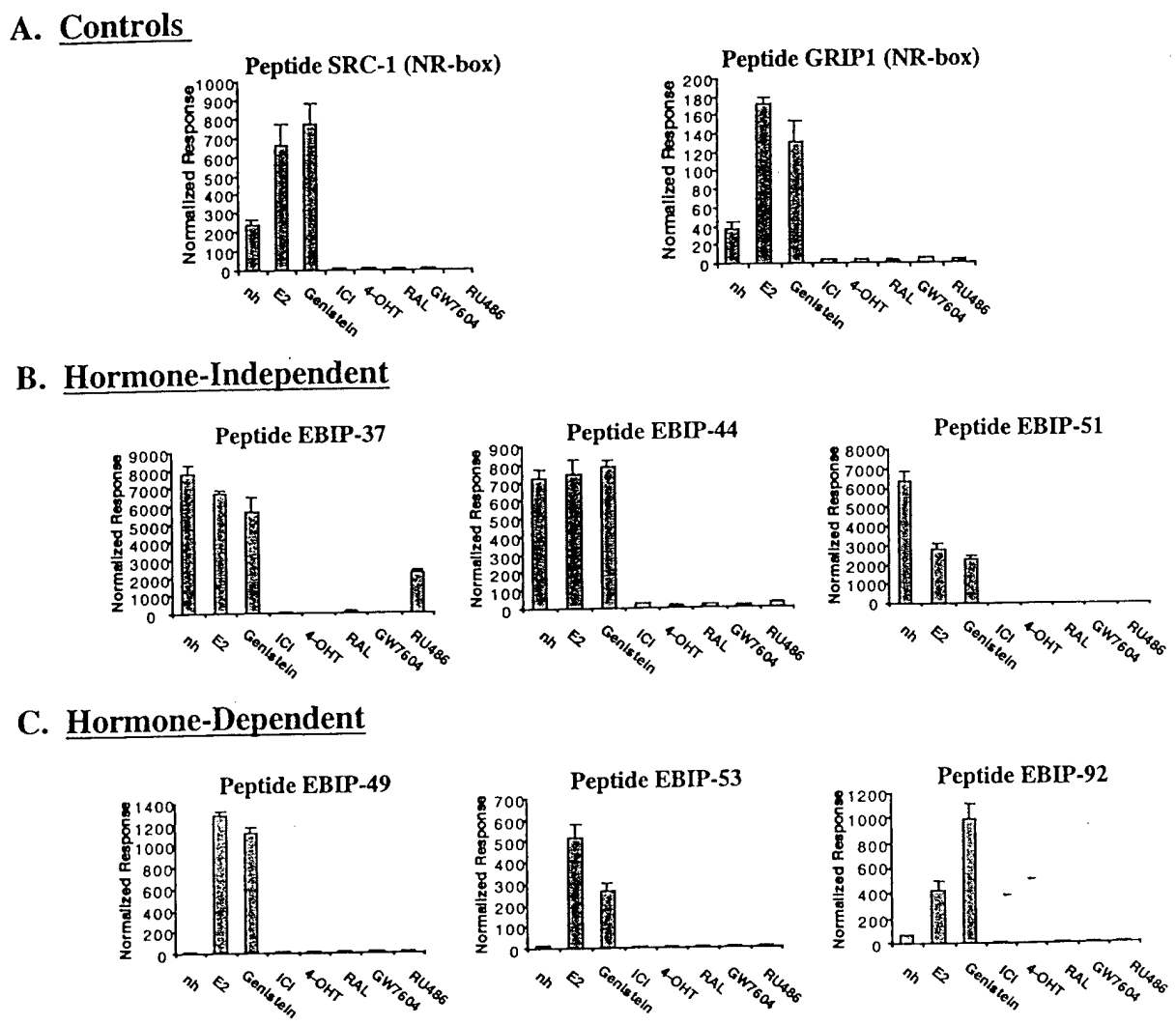


Fig. 2. ERβ-Selective LXXLL Peptides Display Hormone Specificity

Mammalian two-hybrid assays were performed to examine the interactions between the peptides and ERβ in the presence of different ER ligands. HepG2 cells were transiently transfected with the pVP16-ERβ expression vector together with each peptide-Gal4DBD fusion construct, the 5x-GAL4-TATA-Luc reporter, and the pCMV-β-gal control plasmid. After transfection, cells were treated with vehicle (nh) or either 10^{-7} M 17β-estradiol (E_2), ICI182,780 (ICI), 4-hydroxytamoxifen (4OH-T), raloxifene (RAL), GW7604, RU486, or 10^{-6} M genistein. After 24 h cells were harvested and assayed for luciferase activity, and all transfections were normalized for efficiency using the internal pCMV-β-gal control plasmid. Each data point is the average of triplicate determinations, and the average coefficient of variance for each value is <10%. A, The SRC-1 NR-box and GRIP1 NR-box were used as controls. Based upon the pattern of interactions, the peptides were divided into two classes: B, hormone-independent (six peptides) and C, hormone-dependent (nine peptides). The activities of three representative peptides are shown in both parts B and C, and these illustrate the three different patterns of interactions observed in each case.

in the presence of agonist (Fig. 2B). However, these interactions were antagonized by antiestrogen administration. Interestingly, peptide EBIP-51 interacted most efficiently with apo-ERβ, suggesting the existence of cofactors that prefer to associate with ERβ in a constitutive manner (see *Discussion*). The binding of LXXLL motifs to ERβ in the absence of hormone was puzzling, in view of the fact that it was previously observed that the human ERβ does not demonstrate ligand-independent transcriptional activity (11). This result suggests that the aporeceptor may recruit cofactors in some contexts, but that this interaction is not transcriptionally productive. In these studies, we also observed that the antiprogestin RU486 was able to decrease interactions between the peptides and receptor in these assays, a result that agrees with recent reports that RU486 can function as an ERβ antagonist (23).

Nine of the peptide fusions studied were found to interact only with agonist-activated ERβ. Interestingly, within this class, three distinct binding patterns were observed (Fig. 2C). Specifically, peptide EBIP-92 appeared to interact more efficiently with genistein-activated ERβ than that activated by 17β-estradiol, whereas the estradiol-activated receptor interacted equally as well with EPIP-49, and more efficiently with EBIP-53. These data, indicating that genistein and estradiol do not function in the same manner when assayed on ERβ, were interesting in light of the unique functional properties that have recently been ascribed to genistein (8, 24). Similar differences in efficacy were noted when the experiments were repeated over a full range of ligand concentrations (data not shown). These results suggest that estradiol and genistein induce unique conformational changes within ERβ. This hypothesis is supported by recent crystallography studies that showed that ERβ helix 12 (AF-2) assumes a distinct position when occupied by genistein compared with the distinctive agonist position observed for helix 12 of the estradiol-bound ERα (16, 25). If the estradiol-ERα crystallographic data are extrapolated to ERβ, it is possible that genistein-liganded ERβ interacts with cofactors in a different manner than the estradiol-activated receptor or that the former complex recruits a unique coactivator in some settings.

Affinity Does Not Explain the Hormone-Independent Interaction of LXXLL Peptides with ERβ

One of the most interesting classes of peptides identified in this study were those that interacted with ERβ in a constitutive manner but that were unable to interact with the receptor when occupied by antagonists. A trivial explanation for these observed binding characteristics was that these peptides had a higher affinity for ERβ than the peptides that required ligand. Alternatively, these peptides may interact in a unique manner with the coactivator binding pocket within ERβ. These different possibilities were tested using a quantitative phage ELISA. Specifically, the phage stocks of each peptide-expressing clone were titered, and then the binding of different concentrations of phage expressing peptides to ERβ was tested in the presence and absence of estradiol. The results of this analysis, shown in Fig. 3, indicated that even at the lowest input phage concentrations, the hormone-independent peptides maintained their ligand-independent interactions with ERβ; two representative peptides are shown. This was in contrast to the hormone-dependent peptides, which maintained their estradiol-dependent interaction with ERβ throughout the range of phage concentrations (representative example shown in Fig. 3C). Neither the absolute numbers of phage binding

F3

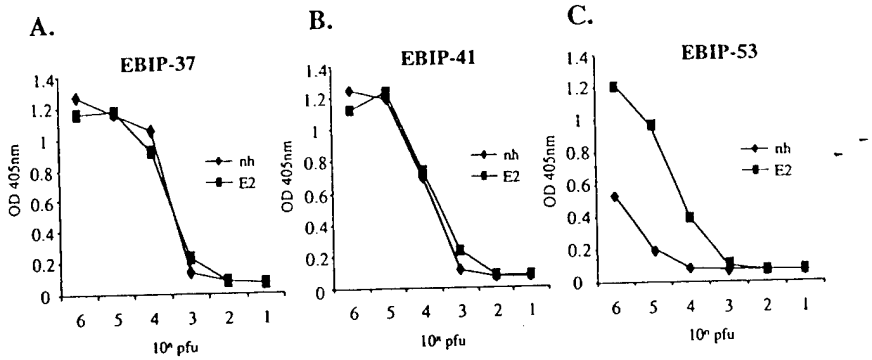


Fig. 3. Affinity Does Not Explain the Hormone-Independent Interaction of LXXLL Peptides with ERβ

The binding of each titered phage clone to ERβ was measured by ELISA. Purified ERβ protein was added to 96-well plates, and 50 μl of each phage stock or serial dilutions were added to individual wells and incubated with the ER target for 1 h at room temperature. The assays were performed in the absence and presence of 10^{-6} M 17β-estradiol. The wells were washed with PBST to remove nonbinding phage and incubated with a horseradish peroxidase-conjugated anti-M13 antibody. Immunocomplexes were detected with ABTS (2′, 2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid) supplemented with 0.05% H_2O_2. The colorimetric change was quantitated by measuring the absorbance at 405 nm. The binding patterns of two representative peptides from the hormone-independent class (A and B), and the binding of one representative peptide from the hormone-dependent class (C) are shown.

to each target nor the apparent binding affinity were significantly different. These results indicate that receptor-binding affinity is not what distinguishes peptides that interact with ERβ in a ligand-dependent manner from those that bind constitutively, but rather these classes of LXXLL-containing peptides interact in different ways with the ERβ-coactivator binding pocket.

Disruption of ERβ Transcriptional Activity by LXXLL-Containing Peptides

The ability of the ERβ-interacting peptides to function as ER subtype-selective antagonists in target cells was next assessed. This was accomplished using transcriptional interference assays in mammalian cells transfected with either ERα or ERβ, together with an empty Gal4DBD vector (pM) or Gal4DBD fusions of the peptides. The EBIP-37, EBIP-41, and EBIP-45 peptides were used in these initial experiments, because they appeared to interact most efficiently with ERβ in the mammalian two-hybrid system (Fig. 1A). Under the conditions of this assay, we noted that the transcriptional activity of ERα was unaffected by overexpression of the ERβ-selective peptides (Fig. 4A). Coexpression of the GRIP1 NR-box sequence (three LXXLL motifs), however, inhibited ERα transcriptional activity by 77%, thus validating the use of LXXLL peptides as antagonists of ER transcriptional responses. When the effect of our peptides on ERβ activity was assessed, we observed that all of the LXXLL sequences functioned as effective antagonists (Fig. 4B). Significantly, coexpression of peptide EBIP-37 with ERβ resulted in 100% inhibition of the transcriptional response, whereas the EBIP-41, EBIP-45, and GRIP-1 (NR-box) peptide fusions produced 85, 70, and 90% inhibition, respectively. Western immunoblotting revealed that expression of the peptides did not alter the cellular levels of ERβ (data not shown). These results indicate that it is possible to target ERβ-coactivator interactions in a selective manner and validate this general approach to develop antagonists of additional members of the nuclear receptor superfamily.

ERβ-Selective LXXLL-Containing Peptides Show Distinct Preferences for Other Nuclear Receptors

One of the major objectives of this study is to develop highly specific inhibitors that can be used to evaluate the relative contributions of the two ER subtypes in estrogen signaling. Consequently, we next examined the ability of the 15 ERβ-selective peptides to interact with other members of the nuclear receptor superfamily. This was accomplished using the mammalian two-hybrid assay to examine interactions of each peptide with 11 different nuclear receptors. Table 1 summarizes the interactions observed between each of the peptides and receptors in the presence of their cognate ligands. No clear pattern emerged from these studies, as each receptor appeared to exhibit distinct peptide binding preferences. Based on these results, it is possible that most of the receptors will eventually be found to display different cofactor preferences. The observation that the androgen receptor (AR) did not interact with any of the peptides tested and that RORα interacted with only two of the 15 peptides suggest that these receptors might have very specific coactivator requirements.

Interestingly, the peptides identified in our screen using unliganded ERβ as bait (EBIP-37, EBIP-41, EBIP-44, EBIP-45), and which interacted with the receptor in an agonist-independent manner in mammalian cells (Fig. 3), were found to interact with most of

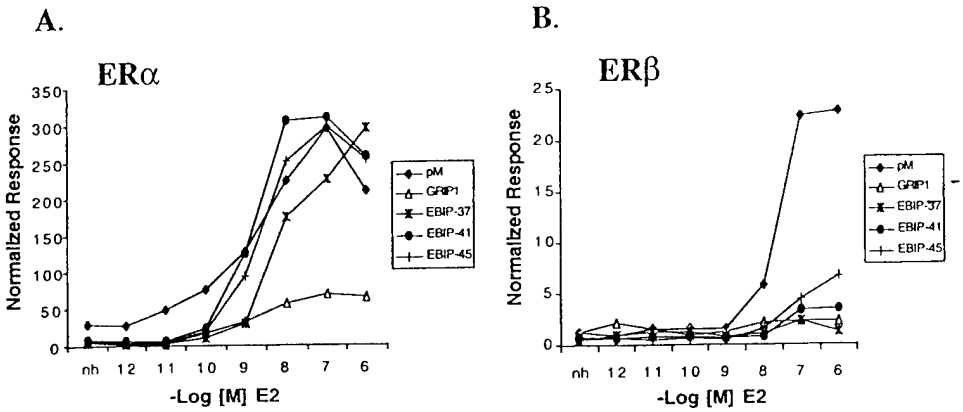


Fig. 4. Disruption of ERβ Transcriptional Activity by LXXLL-Containing Peptides

The effects of three LXXLL-containing peptides (EBIP-37, EBIP-41, and EBIP-45) on ERα and ERβ transcriptional activity were examined. A peptide containing the LXXLL motifs (NR box) of the coactivator GRIP1 was used as a control. HepG2 cells were transiently transfected with either the ERα (A) or ERβ (B) expression vector, together with the empty Gal4DBD vector (pM) or the Gal4DBD-peptide fusion constructs and the 3x-ERE-TATA-Luc reporter and pCMV-β-gal control plasmid. Cells were induced with vehicle (nh) or increasing concentrations (ranging from 10^{-12} M to 10^{-6} M) of 17β-estradiol (E_2) for 24 h, and luciferase assays were performed. Each value was normalized to the β-galactosidase activity. Each data point is the average of triplicate determinations, and the average coefficient of variance for each value is <10%.

Table 1. ERβ-Selective LXXLL-Containing Peptides Show Distinct Preferences for Other Nuclear Receptors

	ERα	ERβ	PR-A	PR-B	GR	AR	RARα	RXRα	TRβ	VDR	RORα	LXR	FXR
SRC-1	+[a]	+	+	+	+	–	+	+	+	+	–	+	+
GRIP1	+	+	+	+	+	–	+	+	+	+	–	+	+
EBIP-37	–	+	+	+	+	–	+	+	+	+	+	+	+
EBIP-41	–	+	–	–	+	–	+	+	+	–	–	+	–
EBIP-44	–	+	+	+	+	–	+	+	+	–	–	+	+
EBIP-45	–	+	+	+	–	–	–	+	+	–	–	+	–
EBIP-49	–	+	–	–	+	–	–	–	–	–	–	–	–
EBIP-51	–	+	–	–	–	–	–	+	+	+	–	+	–
EBIP-53	–	+	+	+	+	–	–	+	+	–	–	+	–
EBIP-56	–	+	–	–	–	–	–	–	–	–	–	–	–
EBIP-60	–	+	+	+	+	–	–	+	–	–	–	+	–
EBIP-66	–	+	+	+	+	–	–	+	–	–	–	–	–
EBIP-70	–	+	–	–	–	–	+	+	+	+	–	+	–
EBIP-76	–	+	–	–	–	–	–	+	+	–	–	+	–
EBIP-87	–	+	+	+	+	–	+	+	+	–	–	+	–
EBIP-92	–	+	–	–	–	–	–	–	–	–	–	–	–
EBIP-96	–	+	+	+	+	–	–	–	+	+	+	+	–

The ability of the ERβ-selective LXXLL motifs to interact with several nuclear receptors was tested in the mammalian-two-hybrid assay (+ denotes interaction; – denotes lack of interaction). Shown here are the interactions that form between the peptides and receptors in the presence of the receptor-specific agonists, with the exception of RORα, which is constitutively active. An interaction (+) was defined by the observation of a statistically significant increase in activity when both the receptor and peptide were coexpressed compared to that present when either was cotransfected with the parent vector of the other. HepG2 cells were transiently transfected with each pVP16-receptor fusion expression vector in combination with each peptide-Gal4DBD fusion construct, the 5x-GAL4-TATA-Luc reporter, and the pCMV-β-gal control plasmid. Each receptor expression construct includes the VP16 activation domain sequence fused 5′ to the entire coding sequence for the human form of the respective receptor, with the exception of pVP16-FXR, which contains the rat FXR homolog. Following transfection, cells were treated with vehicle or the following hormones: 10^{-7} M 17β-estradiol for ERα and ERβ, 10^{-7} M progesterone for PR-A and PR-B, 10^{-7} M dexamethasone for GR, 10^{-6} M 5α-dihydrotestosterone for AR, 10^{-7} M 9-*cis*-retinoic acid for RARα and RXRα, 10^{-7} M T_3 for TRβ, 10^{-7} M 1,25-dihydroxyvitamin D_3 for VDR, 10 μM 22R-hydroxycholesterol for LXR, and 50 μM chenodeoxycholic acid for FXR. After 24-h luciferase assays were performed, and each value was normalized to the β-galactosidase activity, – indicates that no significant increase in activity was observed when the receptor and peptide were coexpressed.

the nuclear receptors tested. The most important result, however, was that EBIP-56 and EBIP-92 interacted exclusively with ERβ and did not interact with any other receptor under the conditions tested. Other peptides, such as EBIP-87 and EBIP-96, were found to interact with multiple receptors. Cumulatively, the results of these studies indicate that it is possible to identify LXXLL-containing peptides that interact in a highly specific manner with ERβ.

Evaluation of the Antagonist Properties of Peptides That Interact in a Specific Manner with ERβ

The antagonist efficacy of the ERβ-specific peptides, EBIP-56 and EBIP-92, was next evaluated. The nuclear receptor interaction regions of most of the well validated coactivators have been shown to contain multiple LXXLL domains, which facilitate the interaction of these proteins with the AF-2 coactivator binding pocket of their targeted receptor (18). Reflecting this observation, we created two-copy Gal4DBD fusions of our peptides. The two LXXLL motifs were separated by sequences corresponding to the linker region between NR-box 2 and NR-box 3 of GRIP1. When expressed in mammalian cells, we observed that while the GRIP1 NR-box sequences inhibited the activity of ERα by 60%, the peptides 2xEBIP-56 and 2xEBIP-92 had no effect on transcriptional response (Fig. 5A). However, when tested on ERβ, it was found that the 2xEBIP-56 and 2xEBIP-92 peptides suppressed estrogen-stimulated transcriptional activity by 82% and 97%, respectively (Fig. 5B). Similar results were obtained using 1xEBIP-56 and 1xEBIP-92, although higher levels of expression of these peptides were required to attain the same degree of antagonism as their dimeric counterparts. Western immunoblotting was used to demonstrate that expression of these peptides did not alter cellular levels of ERβ (data not shown). Thus, ERβ-specific LXXLL-containing peptides can function as potent inhibitors of ERβ transcriptional activity.

F5

ERβ-Specific LXXLL-Containing Peptides Disrupt the Transcriptional Activity of ERα/ERβ Heterodimers

We and others have demonstrated that the transcriptional activity of agonist-activated ERα is significantly greater than ERβ in most cell and promoter contexts (7, 9–11). Not surprisingly, therefore, in cells engineered to produce both ER subtypes, the overall re-

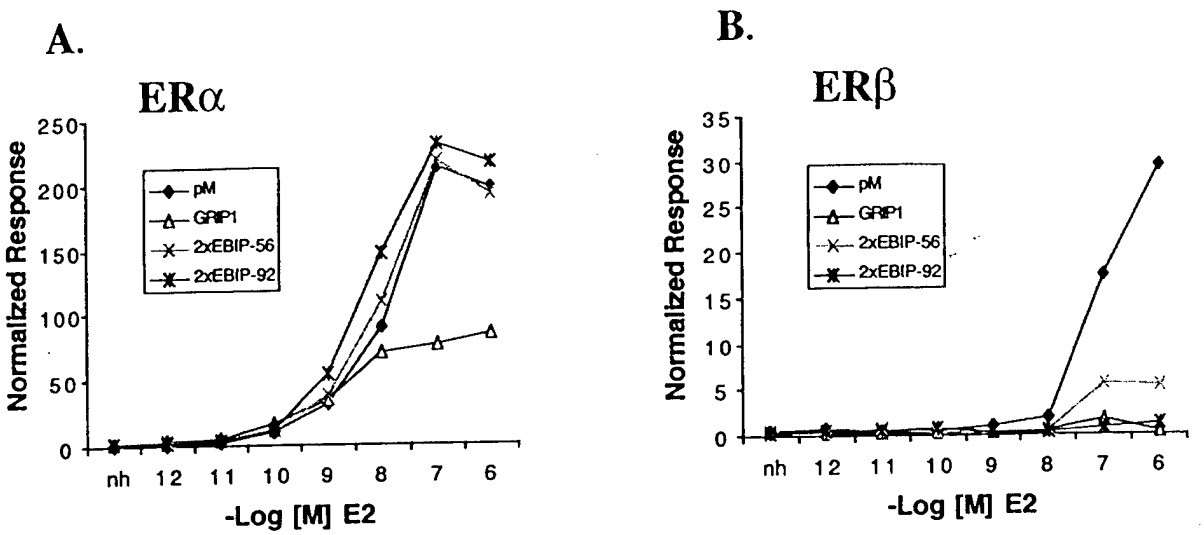


Fig. 5. Evaluation of the Antagonist Properties of Peptides That Interact in a Specific Manner with ERβ

The ability of the ERβ-specific LXXLL peptides EBIP-56 and EBIP-92 to disrupt the transcriptional activities of ERα and ERβ was examined. For these experiments, constructs containing two copies of the peptides (2x-EBIP-56 and 2x-EBIP-92) were used. A peptide containing the three LXXLL motifs (NR-boxes) of the coactivator GRIP1 was used as a control. HepG2 cells were transiently transfected with the ERα (A) or ERβ (B) expression vector together with the empty Gal4DBD vector (pM) or the Gal4DBD-peptide fusion constructs and the 3x-ERE-TATA-Luc and pCMV-β-gal plasmids. After transfection, cells were treated with vehicle (nh) or increasing concentrations (ranging from 10^{-12} M to 10^{-6} M) of 17β-estradiol (E_2) for 24 h, and luciferase assays were performed. Each value was normalized to the β-galactosidase activity. Each data point is the average of triplicate determinations, and the average coefficient of variance for each value is <10%.

sponse to estradiol is reduced. This suggests that one of the functions of ERβ is to modulate ERα transcriptional activity in target cells. At subsaturating concentrations of agonist, ERβ completely suppresses ERα transcriptional activity, whereas no inhibition is observed when the assay is performed in the presence of saturating concentrations of 17β-estradiol (11). These findings suggest that the role of ERβ in estrogen signaling is complex. Previously, we have shown that ERβ is bound constitutively to DNA in the absence of hormone (11). Consequently, at subsaturating concentrations of hormone, the receptor is capable of competitively inhibiting the activity of ERα homodimers and ERα/ERβ heterodimers by blocking their ability to interact with target gene promoters. Under saturating concentrations of hormone, we have proposed that ERα/ERβ heterodimers form, and that the functional properties of this complex are similar to that of an ERα homodimer. However, without a specific ERβ antagonist, it has not been possible to prove that the heterodimeric ERα/ERβ complex was functionally active in cells.

The identification of specific peptide antagonists of ERβ has enabled us to evaluate the functional significance of ERα/ERβ heterodimers. This was accomplished by determining the impact of expressing the 2xEBIP-92 antagonist in cells and examining its impact on ERα and ERβ-mediated transcriptional activity. The results of this analysis are shown in Fig. 6. As observed previously, expression of 2x-EBIP-92 in cells had no effect on ERα transcriptional activity, whereas it completely suppressed ERβ activity. Importantly, however, in cells expressing both receptor subtypes it was demonstrated that the ERβ-specific antagonist, 2xEBIP-92, was capable of significantly reducing the transcriptional activity of the ERα/ERβ heterodimer. To rule out the possibility that in the context of the heterodimer, the ERβ-specific peptides may interact directly with ERα we used mammalian two-hybrid assays to assess the interaction of the heterodimeric complexes with a subset of the EBIPs. For this purpose, we created an ERβ mutant that disrupts AF-2 but that has no effect on the receptor's ligand binding characteristics. As shown in Fig. 7A, an ERβ-VP16, but not an ERβ-3X-VP16, chimera was able to interact with the LXXLL peptides when tested in the two-hybrid assay. Since ERβ-3x heterodimerizes with ERα in a manner that was indistinguishable from ERβ it was possible to use this mutant to test the interaction of the ERβ-interacting peptides with the ER α/β heterodimer (11). First, we demonstrated that expression of ERα in target cells had no effect on the ability of ERβ to interact with EBIPs 37, 41, 56, or 92 (Fig. 7B). Although it would be difficult to quantitate the amount of heterodimer formed under the conditions of this assay, the results suggest that ERβ specificity is maintained in this context. The most important result, however, was that in cells expressing ERα, the ERβ-3x-VP16 chimera was inactive in the two-hybrid assay (Fig. 7B). Given the characteristics of the ERβ-3x chimera, a positive interaction in the two-hybrid assay would only have been possible if the peptides were able to inter-

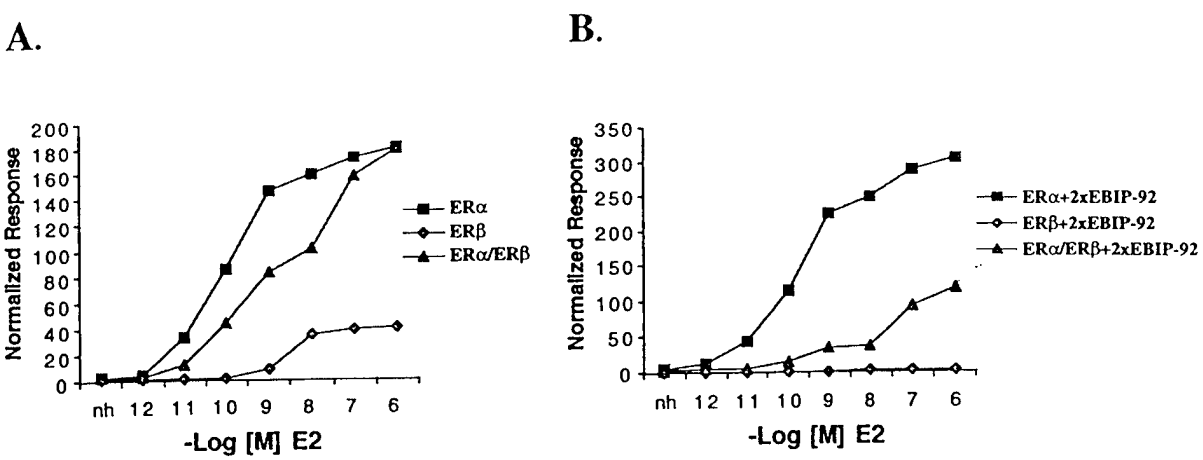


Fig. 6. ERβ-Specific LXXLL Containing Peptides Disrupt the Transcriptional Activity of ERα/ERβ Heterodimers

HepG2 cells were transiently transfected with either the ERα or ERβ expression vectors, or equal quantities of both vectors together with the pM (Gal4DBD) empty vector (A) or the 2XEBIP-92 peptide-Gal4DBD fusion construct (B) and the 3x-ERE-TATA-Luc and pCMV-β-gal plasmids. Cells were induced with vehicle (nh) or increasing concentrations (ranging from 10^{-12} M to 10^{-6} M) of 17β-estradiol (E_2) for 24 h, and luciferase assays were performed. Each value was normalized to the β-galactosidase activity. Each data point is the average of triplicate determinations, and the average coefficient of variance for each value is <10%.

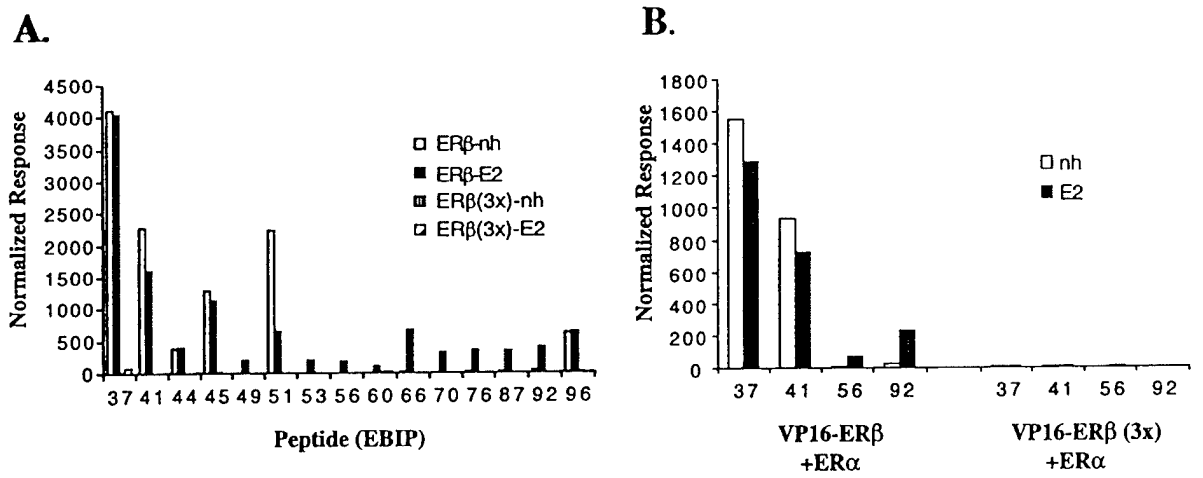


Fig. 7. The Specificity of the ERβ-Interacting Peptides Is Maintained within the Context of an ERα/ERβ Heterodimer

A, Mammalian two-hybrid assays were performed to examine the interactions between the peptides and ERβ or ERβ(3x). ERβ(3x) contains three point mutation introduced into AF-2, which nullify the AF-2 activity of the receptor. HepG2 cells were transiently transfected with the pVP16-ERβ or pVP16-ERβ(3x) expression vector together with each peptide-Gal4DBD fusion construct, the 5x-GAL4-TATA-Luc reporter, and the pCMV-β-gal control plasmid. B, Mammalian two-hybrid assays were performed to examine the interactions between the peptides and ERα/ERβ heterodimers or ERα/ERβ(3x) heterodimers. The interaction of four representative peptides are shown. HepG2 cells were transiently transfected with the pVP16-ERβ or pVP16-ERβ(3x) expression vector and an ERα expression vector together with each peptide-Gal4DBD fusion construct, the 5x-GAL4-TATA-Luc reporter, and the pCMV-β-gal control plasmid. After transfection, cells were treated with vehicle (nh) or 10^{-7} M 17β-estradiol (E_2). After 24 h, cells were harvested and assayed for luciferase activity, and all transfections were normalized for efficiency using the internal pCMV-β-gal control plasmid. Each data point is the average of triplicate determinations, and the average coefficient of variance for each value is <10%.

act with the coactivator binding pocket of ERα. We conclude from this experiment that even within the context of an ERα/ERβ heterodimer that the ER-interacting characteristics of the EBIPs are maintained. Therefore, the inhibitory effects of 2xE-BIP-92 on the ERα/β heterodimer is mediated by blocking ERβ function. Thus, although it has been shown previously that ERα and ERβ preferentially form heterodimers when coexpressed (26, 27), we now demonstrate ERβ contributes in a positive manner to the overall activity of the complex under agonist saturating conditions.

Comparison of the Amino Acid Sequences of the ERβ-Interacting Peptides

Alignment of the three classes of ERβ-interacting peptides identified (hormone-independent, hormone-dependent, and ERβ-specific) was next performed (Table 2). Surprisingly, minimal homology was observed outside of the conserved LXXLL motif between members within the same class. However, we observed that the two ERβ specific peptides, EBIP-56 and EBIP-92, contain a tryptophan residue at position −5 that was not found in any of the other 68 ERβ-interacting peptides that were isolated in the primary screen. Therefore, we postulated that the tryptophan influenced the specificity of the peptide-ERβ interactions. To test this hypothesis, the tryptophan residue in the EBIP-92 peptide was converted to glutamine (an amino acid found at this position in many of the peptides identified). Analysis using a mammalian two-hybrid revealed that while the wild-type EBIP-92 interacted with ERβ in a hormone-dependent manner, a variant peptide in which the tryptophan residue at position −5 was mutated was unable to interact with ERβ (data not shown). These studies demonstrate the importance of the sequences surrounding LXXLL motifs in determining receptor selectivity and suggest that it may be possible to use site-directed mutagenesis to optimize the interactions of the peptides identified with their protein targets.

Table 2. Comparison of the Amino Acid Sequences of the ERβ-Interacting Peptides

	−3−2−1	LXXLL	+1+2+3
ERβ-selective peptides			
Hormone-independent			
Peptide EBIP-37	TGGGVSL	**LLHLL**	NTEQGES
Peptide EBIP-41	RRDDFPL	**LISLL**	KDGALSQ
Peptide EBIP-44	YGLKMSL	**LESLL**	REDISTV
Peptide EBIP-45	MSYDMLS	**LYPLL**	TNSLLEV
Peptide EBIP-51	FPAEFPL	**LTYLL**	ERQGMDE
Peptide EBIP-96	VESEFPY	**LLSLL**	GEVSPQP
Hormone-dependent			
Peptide EBIP-49	VSSEGRL	**LIDLL**	VDGQQSE
Peptide EBIP-53	DTPQSPL	**LWGLL**	SSDRVEG
Peptide EBIP-60	GGTQDGY	**LWSLL**	TGMPEVS
Peptide EBIP-66	SLPEEGF	**LMKLL**	TLEGDAE
Peptide EBIP-70	VMGNNPI	**LVSLL**	EEPSEEP
Peptide EBIP-76	VLVEHPI	**LGGLL**	STRVDSS
Peptide EBIP-87	QTPL	**LEQLL**	TEHIQQG
ERβ-specific peptides			
Peptide EBIP-56	GSWQDSL	**LLQLL**	NRTELMA
Peptide EBIP-92	SVWPGPE	**LLKLL**	SGTSVAE

The ERβ-selective peptides were divided into two classes: hormone-independent and hormone-dependent. Also shown are the sequences of the ERβ-specific peptides EBIP-56 and EBIP-92, which constitute a third class of ERβ-interacting peptides. The conserved tryptophan at position −5 relative to the LXXLL motif in these two sequences is shown in *bold*.

DISCUSSION

Development of ER Peptide Antagonists

The most important outcome of this series of studies was the identification of highly potent, specific ERβ antagonists. Using these peptides, it is possible to efficiently inhibit ERβ transcriptional activity by disrupting interactions between the receptor and cellular coactivators. These reagents are important tools that will facilitate an evaluation of the role of this ER subtype in estrogen signaling. For instance, we have used these peptides to demonstrate that ERα/ERβ heterodimeric complexes can form within cells, and that ERβ contributes in a positive manner to the overall activity of the estrogen-activated complex.

Recently, it has been suggested that the two ER subtypes may oppose the actions of each other in target organs. Although controversial, this hypothesis is supported by the observation that the βERKO mouse displays epithelial hyperplasia in the prostate and bladder (14), an increase in bone mineral content (28), and an increased responsiveness to estrogen in the uterus (29), reflecting possibly an enhancement of ERα-mediated transcriptional activity. Using an appropriate delivery system, it may be possible to antagonize ERβ action using the receptor-specific peptides and test directly the hypothesis that ERβ functions as an ERα modulator in some tissues.

It has recently been shown that both ER subtypes are expressed in breast tumors (30–32) and that ERβ expression is up-regulated in tumors that have developed tamoxifen resistance (32). Thus, there is an unmet medical need to develop novel ER antagonists as 1) potential breast cancer therapeutics and 2) tools to specifically define the role of ERβ in breast cancer cell biology. The finding that none of the LXXLL-containing sequences in this study interact with antiestrogen-liganded receptor suggests that suitably formulated ER peptide antagonists could be coadministered with tamoxifen to completely block estrogen-stimulated proliferative pathways in the breast, using two mechanistically distinct modes of antagonism. Recent studies provide evidence that tamoxifen resistance in breast tumors may arise from the up-regulation of coactivator proteins, which may permit cells to recognize tamoxifen as an agonist and growth stimulant (22). The identification of peptides that disrupt receptor-coactivator interactions provides a novel mechanism by which the mitogenic actions of activated ER can be blocked in both antiestrogen-responsive and -resistant breast cancer cells. Theoretically, the peptide antagonists that we have identified could be developed as second line pharmaceutical treatments for ER-positive, tamoxifen-refractory tumors.

Previous studies in our laboratory (20) reported the identification of the ERβ selective peptide 293. However, while the peptide displays selectivity for ERβ over ERα, 293 was found to interact with many of the other nuclear receptors. In this study our goal was to

develop peptides that interacted in a completely specific manner with ERβ, which was accomplished in the discovery of peptides EBIP-56 and EBIP-92. A similar study was recently reported (33) in which a panel of LXXLL-containing peptides were identified that demonstrated selectivity for ERβ over thyroid hormone receptor (TR). However, the authors of that study indicated that most of their ERβ-interacting peptides cross-react with ERα. Thus, EBIP-56 and EBIP-92 represent the only reagents available that can be used to specifically inhibit ERβ transcriptional activity.

Ligand-Independent Recruitment of LXXLL Motifs

One of the most important findings of this study was that the unliganded ERβ is capable of recruiting many of the LXXLL peptides. Interestingly, studies with ERα showed that LXXLL-containing sequences were capable of a low but significant basal level of interaction in the absence of hormone (20). These results suggested that a fraction of the ERα molecules in a cell might reside in an active conformation, thus permitting recruitment of LXXLL motifs in the absence of receptor agonists. This may explain why ERα can activate transcription in some contexts in the absence of hormone. Surprisingly, although apo-ERβ is capable of binding several different LXXLL-containing peptides, this form of the receptor does not activate transcription in the absence of agonist (11). Consistent with this observation, we have shown using *in vitro* protein-protein interaction studies that ERβ, but not ERα, can bind to GRIP1 in the absence of ligand (our unpublished results). One possibility is that the ERβ aporeceptor is present in an inhibitory complex containing both coactivators and corepressors, and that the binding of hormone enhances the functionality of associated activators and promotes the dissociation of repressor proteins. Alternatively, unliganded ERβ may bind to some cofactors in a manner that is not transcriptionally productive. An activity of this nature has not yet been demonstrated for ERβ; however, it has been shown that unliganded peroxisome proliferator activated receptor-γ (PPARγ) interacts with the coactivator PGC-1 (PPARγ coactivator 1, a protein that has no apparent coactivator activity), and this protein is responsible for recruiting SRC-1 when agonist is added (34).

The ability of nuclear receptors to interact with LXXLL motifs in their apo- state raises the possibility that ligand regulation of coactivator recruitment may have evolved to enable receptor activity to respond to changes in cellular homeostasis. Consistent with this hypothesis is the observation that several orphan receptors [estrogen-receptor-related proteins (ERR1, ERR2, and ERR3)] that have not yet been shown to require ligands bind SRC-1, GRIP1, or activator of thyroid receptor (ACTR) in a ligand-independent manner (35, 36). Similarly, the orphan receptor 1/ retinoid X receptor (OR1/RXR) heterodimer is capable of ligand-independent cofactor recruitment (37). Recent studies have also illustrated that ligand-independent signaling pathways can result in activation of ERβ by promoting agonist-independent coactivator binding (38). These observations suggest that the general mechanisms of hormone-dependent and independent transcriptional activation by nuclear receptors may be similar, and that in some cases the role of ligand may be as a catalyst, but not as a required part of receptor activation. Thus, ERβ may be a receptor whose state of evolution is intermediate between the orphan receptors and the more classical steroid receptors.

Nuclear Hormone Receptors Have Distinct Preferences for LXXLLs

A recurring theme in these studies is that nuclear receptors have distinct preferences for LXXLL motifs. Previous work in our laboratory using peptide display has demonstrated that the sequences flanking the core LXXLL domain are important determinants of receptor selectivity (20). Mutagenesis studies have also been used to identify residues important for both receptor binding affinity and specificity (18, 39). McInerney *et al.* demonstrated that of the three helical LXXLL-containing regions of SRC-1, a single helical domain was sufficient for ER activation, whereas a combination of two distinct helical regions were required for PR, TR, retinoic acid receptor (RAR), and PPARγ actions (40). These studies indicate that different receptors can interact with the same cofactor in different ways.

To complement these previous studies, we observed that peptide EBIP-37 (identical to an LXXLL motif in RIP140) interacted selectively with ERβ, but not ERα. Therefore, since RIP140 can bind to and repress the transcriptional activities of both ERα and ERβ (Ref. 41 and our unpublished data), it is likely that each receptor subtype utilizes distinct LXXLL motifs within this factor, enabling them to bind. The observation that each of the receptors examined in our study displayed a unique pattern of interaction with LXXLL peptides also provides evidence that the receptors may bind different coactivators, or alternatively, recruit the same factors by utilizing distinct binding regions. It is likely therefore, that it will be possible to develop LXXLL-containing antagonists for many of the nuclear receptors. It was surprising, given the structural conservation among the nuclear receptors and associated cofactors, that peptides could be identified which block these interactions in a highly specific manner. However, given that it has been possible to develop specific ERβ antagonists using this approach, we believe that it will be feasible to identify inhibitors of a wide variety of transcription factors by interfering with specific protein-protein interactions.

MATERIALS AND METHODS

Biochemicals

DNA restriction and modification enzymes were obtained from Roche Molecular Biochemicals (Indianapolis, IN), New England Biolabs, Inc. (Beverly, MA), or Promega Corp. (Madison, WI). PCR reagents were obtained from Perkin Elmer Corp. (Norwalk, CT) or Promega Corp. 17β-Estradiol, genistein, 4-hydroxytamoxifen, 9-*cis*-retinoic acid, dexamethasone, 5α-dihydrotestosterone, T_3, progesterone, 22R-hydroxycholesterol, and chenodeoxycholic acid were purchased from Sigma (St. Louis, MO). RU486 was a gift from Ligand Pharmaceuticals, Inc. (San Diego, CA). The estrogen receptor antagonist ICI 182,780 was a gift from Dr. Alan Wakeling (Zeneca Pharmaceuticals, Macclesfield, UK). Raloxifene was a gift from Dr. Eric Larsen (Pfizer, Inc., Groton, CT). GW7604 was a gift from Dr. Tim Willson (Glaxo-Wellcome, Research Triangle Park, NC); and 1,25-dihydroxyvitamin D_3 was purchased from Duphar Pharmaceuticals (Daweesp, The Netherlands). The mouse monoclonal anti-Gal4DBD antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The rabbit polyclonal ERβ antibody was a gift from Dr. Geoffrey Greene (University of Chicago, Chicago, IL). Secondary antibodies, Hybond-C extra transfer membranes, and ECL reagents were purchased from Amersham Pharmacia Biotech (Arlington Heights, IL).

Affinity Selection of ERβ-Binding Peptides

Baculovirus-expressed human ERβ (amino acids 1–477) was purchased from PanVera Corp. (Madison, WI). ERβ (4 pmol) was added to 100 μl of $NaHCO_3$, pH 8.5, in single wells of a 96-well Immulon 4 plate (Dynex Technologies, Inc.). The protein was then incubated in the absence or presence of 10^{-6} M 17β-estradiol overnight at 4 C. A duplicate well containing BSA alone was used as a control. The wells were blocked with 150 μl of 0.1% BSA in $NaHCO_3$ for 1 h at room temperature and then washed five times with PBST [137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.3, 0.1% Tween 20). Twenty five microliters of the phage library ($>10^{10}$ phage) were preincubated on ice for 1 h in 125 μl PBST, 0.1% BSA, and 10^{-6} M 17β-estradiol or vehicle. The phage library was added to the wells, and the plate was sealed and incubated at room temperature for 8 h with gentle agitation. The wells were washed five times with PBST to remove nonbinding phage. The binding phage were eluted with 100 μl of 50 mM glycine-HCl, pH 2.0 (prewarmed to 50 C), and subsequently eluted with 100 μl of 100 mM ethanolamine, pH 11.0. The first eluant was neutralized with 200 μl of 200 mM Na_2HPO_4, pH 8.5, before being combined with the second eluant. The bound phage were amplified in DH5αF′ cells for 6 h and recovered by centrifugation. The amplified phage were used for subsequent rounds of panning. Three rounds of panning were performed. The enrichment of ERβ binding phage in each round of panning was confirmed by ELISA. Individual phage clones were purified after the third round of panning. The single-stranded phage DNA was isolated from each clone, and the peptide sequences were determined by DNA sequencing.

ELISA

Purified ERβ protein (0.4 pmol) was added to 96-well Immulon 4 plates as detailed above. Fifty microliters of each purified phage were added to an individual well and incubated with the ER target for 1 h at room temperature. The assays were performed in the absence and presence of 10^{-6} M of various ER ligands. The wells were washed five times with PBST to remove nonbinding phage. The binding of each peptide to full-length ERα (provided by Panvera Corp.) in the presence of various ER ligands was also tested in this assay. A horseradish peroxidase-conjugated anti-M13 antibody (Amersham Pharmacia Biotech) was diluted 1:5000 in PBST, 100 μl of the mixture was added to each well, and the solutions were incubated for 1 h at room temperature. The wells were washed five times with PBST, and immunocomplexes were detected with ABTS (2′,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid) supplemented with 0.05% H_2O_2. The colorimetric change was quantitated by measuring the absorbance at 405 nm on a plate reader (Multiskan MS; Labsystems, Marlboro, MA).

Plasmids

The Gal4DBD-peptide fusions were constructed as follows. The peptides were excised from the mBAX phage vectors with *Xba*I and *Xho*I. The parent pMsx vector (20) (containing the Gal4DBD) was digested with *Sal*I and *Xba*I. The peptides were then ligated in frame to the pMsx vector, creating Gal4DBD-peptide fusion constructs. The constructs containing two copies of the LXXLL-containing peptides (2x-EBIP-56 and 2x-EBIP-92) were created as follows: pM-EBIP-56 and pM-EBIP-92 were digested with *Xba*I. The linker region between the second and third LXXLL motifs within the GRIP1 cDNA was amplified by PCR, digested with *Nhe*I and *Xba*I, and ligated into pM-EBIP-56 and pM-EBIP-92, at the 3′ of the peptide. These vectors were then digested with *Sal*I and *Xba*I and a second peptide was inserted into these sites 3′ to the GRIP1 linker. The construction of pM-SRC-1 (NR-box) and pM-GRIP1 (NR-box) has been described previously (20).

The mammalian expression plasmid for the peptide EBIP-92 mutant was constructed by site-directed mutagenesis as follows. The pM-EBIP-92 vector was used as the template, and a point mutation in the conserved tryptophan residue was created using PCR-based oligonucleotide-directed mutagenesis, according to the manufacturer's protocol (Stratagene, La Jolla, CA). The sequences of the oligonucleotides used for PCR were 5′-CTCGAGAAGTGT-TGAGCCGGGTCCGGAGCTGCTTAAGCTGCTGTCGGGGA-CGAGTGTGGCGGAG (forward) and 3′-CTCCGCCACAC-TCGTCCCCGACAGCAGCTTAAGCAGCTCCGGACCCGGC-TCAACACTTCTCGAG (reverse).

pVP16ERα, pVP16ERβ, pVP16RARα, and pVP16RXRα have been described previously (20). VP16GR, VP16PR-A, VP16PR-B, and VP16AR expression plasmids were gifts from J. Miner (VP16GR), D. X. Wen (VP16PR-A and VP16PR-B), and K. Marschke (VP16AR) (Ligand Pharmaceuticals, Inc., San Diego, CA). VP16VDR was a gift of J. W. Pike (University of Cincinnati, Cincinnati, OH), and the VP16TRβ expression plasmid (pCMX-VP-F-hTRβ) was provided by D. D. Moore (Baylor College of Medicine, Houston, TX). pVP16RORα-LBD was a gift from A. R. Means (Duke University Medical Center, Durham, NC). The cDNAs for the human liver X receptor (LXR) and rat farnesoid X receptor (FXR) were provided by D. J. Mangelsdorf (University of Texas, Dallas, TX). pVP16LXR, and pVP16FXR were created as described previously for the other nuclear receptor VP16 fusions (20).

The mammalian expression plasmids for ERα (pRST7ER) and ERβ (pRST7ERβ) have been described previously (11, 42). The reporter 5x-GAL4-TATA-Luc (a gift from Dr. Xiao-Fan Wang, Duke University Medical Center) contains five palindromic copies of the GAL4 transcription factor response element cloned into pGL2-TATA-Inr (Stratagene). The 3x-ERE-TATA-Luc reporter contains three copies of the vitellogenin ERE (43).

All of the PCR-based constructs were sequenced to verify the accuracy of the amplified sequences.

Cell Culture and Transient Transfection Assays

HepG2 cells were maintained in MEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% FCS (Life Technologies, Inc.), 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate. Cells were plated in 24-well plates (coated with gelatin for transfections of HepG2 cells) 24 h before transfection. DNA was introduced into the cells using lipofectin (Life Technologies, Inc.). Triplicate transfections were performed using 3 μg of total DNA. In standard mammalian two-hybrid assays, 1,500 ng of reporter (5x-GAL4-TATA-Luc), 500 ng of receptor-VP16 fusion, 500 ng of pM (Gal4DBD)-peptide fusion constructs, 100 ng of the pCMV-βgal normalization vector (44), and 400 ng of the control vector pBSII-KS (Stratagene) were used. For receptor disruption studies, 1,500 ng of reporter (3x-ERE-TATA-Luc), 250 ng of receptor (either pRST7ERα or pRST7ERβ), 1000 ng of pM-peptide fusion constructs or the parent pM vector, 100 ng of pCMV-βgal, and 150 ng of pBSII-KS were used. Cells were incubated with the DNA/lipofectin mix for 3 to 6 h and then washed with PBS and the transfection mix was replaced with phenol red-free MEM containing 10% charcoal-stripped FCS (HyClone Laboratories, Inc., Logan, UT). The receptor ligands were added to the cells 20–24 h before the assays. Luciferase and β-galactosidase assays were performed as described previously (45). All experiments were repeated a minimum of three times.

Acknowledgments

We thank Drs. D. J. Mangelsdorf, K. Marschke, A. R. Means, J. Miner, D. D. Moore, M. G. Parker, J. W. Pike, X.-F. Wang, and D. X. Wen for their generous gifts of plasmids. We would also like to thank Dr. Geoffrey Greene for the rabbit polyclonal ERβ antibody. We are also grateful to members of our laboratory for critical review of the manuscript and for their help with the phage display technology.

Received April 10, 2000. Revision received July 24, 2000. Accepted August 15, 2000.

Address requests for reprints to: Dr. Donald P. McDonnell, Department of Pharmacology and Cancer Biology, Duke University Medical Center, P.O. Box 3813, Durham, North Carolina 27710. E-mail: mcdon016@acpub.duke.edu.

J.M.H. is supported by a Predoctoral Fellowship (DAMD17-97-1-7277) and C.-Y.C. by a postdoctoral fellowship (DAMD17-99-1-9173) from the US Army Medical Research Acquisition Activity (USAMRAA). This work was supported by a National Institute of Health Grant DK-48807 (to D.P.M.).

REFERENCES

1. Evans RM 1988 The steroid and thyroid receptor superfamily. Science 240:889–895
2. Tsai M, O'Malley BW 1994 Molecular mechanisms of action of steroid/thyroid receptor superfamily members. Annu Rev Biochem 63:451–486
3. Oñate SA, Tsai, S-Y, Tsai M-J, O'Malley BW 1995 Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. Science 270: 1354–1357
4. Hong H, Kohli K, Trivedi A, Johnson DL, Stallcup MR 1996 GRIP1, a novel mouse protein that serves as a transcriptional coactivator in yeast for the hormone binding domains of steroid receptors. Proc Natl Acad Sci USA 93:4948–4952
5. Shibata H, Spencer TE, Oñate SA, Jenster G, Tsai S-Y, Tsai M-J, O'Malley BW 1997 Role of co-activators and co-repressors in the mechanism of steroid/thyroid receptor action. Recent Prog Horm Res 52:141–164
6. Kuiper GGJM, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson J-A 1996 Cloning of a novel estrogen receptor expressed in rat prostate and ovary. Proc Natl Acad Sci USA 93:5925–5930
7. Mosselman S, Polman J, Dijkema R 1996 ERβ: identification and characterization of a novel human estrogen receptor. FEBS Let 392:49–53
8. Kuiper GGJM, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S, Gustafsson J-A 1997 Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β. Endocrinology 138:863–870
9. Tremblay GB, Tremblay A, Copeland NG, Gilbert DJ, Jenkins NA, Labrie F, Giguere V 1997 Cloning, chromosomal localization, and functional analysis of the murine estrogen receptor β. Mol Endocrinol 11:353–365
10. Cowley SM, Parker MG 1999 A comparison of the transcriptional activation by ERα and ERβ. J Steroid Biochem Mol Biol 69:165–175
11. Hall JM, McDonnell DP 1999 The estrogen receptor β-isoform (ERβ) of the human estrogen receptor modulates ERα transcriptional activity and is a key regulator of the cellular response to estrogens and antiestrogens. Endocrinology 140:5566–5578
12. Couse JF, Curtis Hewitt S, Bunch DO, Sar M, Walker VR, Davis BJ, Korach KS 1999 Postnatal sex reversal of the ovaries in mice lacking estrogen receptors α and β. Science 286:2328–2331
13. Couse JF, Korach KS 1999 Estrogen receptor null mice: what have we learned and where will they lead us? Endocr Rev 20:358–417
14. Krege JH, Hodgin JB, Couse JF, Enmark E, Warner M, Mahler JF, Sar M, Korach KS, Gustafsson J-A, Smithies O 1998 Generation and reproductive phenotypes of mice lacking estrogen receptor β. Proc Natl Acad Sci USA 95:15677–15682
15. Sun J, Meyers MJ, Fink BE, Rajendran R, Katzenellenbogen JA, Katzenellenbogen BS 1999 Novel ligands that function as selective estrogens or antiestrogens for estrogen receptor-α or estrogen receptor-β. Endocrinology 140:800–804
16. Brzozowski AM, Pike AC, Dauter Z, Hubbard RE, Bonn T, Engstrom O, Ohman L, Greene GL, Gustafsson J-A, Carlquist M 1997 Molecular basis of agonism and antagonism in the oestrogen receptor. Nature 389:753–758
17. Feng W, Ribeiro RC, Wagner RL, Nguyen H, Apriletti JW, Fletterick RJ, Baxter JD, Kushner PJ, West BL 1998 Hormone-dependent coactivator binding to a hydrophobic cleft on nuclear receptors. Science 280:1747–1749
18. Heery DM, Kalkhoven E, Hoare S, Parker MG 1997 A signature motif in transcriptional co-activators mediates binding to nuclear receptors. Nature 387:733–736
19. Shiau AK, Barstad D, Loria PM, Cheng L, Kushner PJ, Agard DA, Greene GL 1998 The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. Cell 95:927–937
20. Chang C-Y, Norris JD, Gron H, Paige LA, Hamilton PT, Kenan DJ, Fowlkes D, McDonnell DP 1999 Dissection of the LXXLL nuclear receptor-coactivator interaction motif using combinatorial peptide libraries: discovery of peptide antagonists of estrogen receptors α and β. Mol Cell Biol 19:8226–8239
21. Sparks AB, Adey NB, Cwirla S, Kay BK 1996 Screening phage-displayed random peptide libraries. In: Kay BK, Winter J, McCafferty J (eds) Phage Display of Peptides and Proteins: A Laboratory Manual. Academic Press, Inc., San Diego, CA, pp 227–253
22. Lavinsky RM, Jepsen K, Heinzel T, Torchia J, Mullen T-M, Schiff R, Del-Rio AL, Ricote M, Ngo S, Gemsch J,

Hilsenbeck SG, Osborne CK, Glass CK, Rosenfeld MG, Rose DW 1998 Diverse signaling pathways modulate nuclear receptor recruitment of N-CoR and SMRT complexes. Proc Natl Acad Sci USA 95:2920–2925
23. Zou A, Marschke KB, Arnold KE, Berger EM, Fitzgerald P, Mais DE, Allegretto EA 1999 Estrogen receptor beta activates the human retinoic acid receptor α-1 promoter in response to tamoxifen and other estrogen receptor antagonists, but not in response to agonists. Mol Endocrinol 13:418–430
24. Barkhem T, Carlsson B, Nilsson Y, Enmark E, Gustafsson J-A, Nilsson S 1998 Differential response of estrogen receptor α and estrogen receptor β to partial estrogen agonists/antagonists. Mol Pharmacol 54:105–112
25. Pike ACW, Brzozowski AM, Hubbard RE, Bonn T, Thorsell A-G, Engstrom O, Ljunggren J, Gustafsson J-A, Carlquist M 1999 Structure of the ligand-binding domain of oestrogen receptor β in the presence of a partial agonist and a full antagonist. EMBO J 18:4608–4618
26. Cowley SM, Hoarse S, Mosselman S, Parker MG 1997 Estrogen receptors α and β form heterodimers on DNA. J Biol Chem 272:19858–19862
27. Pettersson K, Grandien K, Kuiper GGJM, Gustafsson, J-A 1997 Mouse estrogen receptor β forms estrogen response element-binding heterodimers with estrogen receptor α. Mol Endocrinol 11:1486–1496
28. Windahl SH, Vidal O, Andersson G, Gustafsson J-A, Ohlsson C 1999 Increased cortical bone mineral content but unchanged trabecular bone mineral density in female ERβ (−/−) mice. J Clin Invest 104:895–901
29. Weihua Z, Saji S, Makinen S, Cheng G, Jensen EV, Warner M, Gustafsson J-A 2000 Estrogen receptor (ER) β, a modulator of ERα in the uterus. Proc Natl Acad Sci USA 97:5936–5941
30. Dotzlaw H, Leygue E, Watson PH, Murphy LC 1996 Expression of estrogen receptor-β in human breast tumors. J Clin Endocrinol Metab 82:2371–2374
31. Leygue E, Dotzlaw H, Watson PH, Murphy LC 1998 Altered estrogen receptor α and β messenger RNA expression during human breast tumorigenesis. Cancer Res 58:3197–3201
32. Speirs V, Parkes AT, Kerin MJ, Walton DS, Carleton PJ, Fox JN, Atkin SL 1999 Coexpression of estrogen receptor α and β: poor prognostic factors in human breast cancer? Cancer Res 59:525–528
33. Northrup JP, Nguyen D, Piplani S, Olivan SE, Kwan ST-S, Go NF, Hart CP, Schatz PJ 2000 Selection of estrogen receptor β-, thyroid hormone receptor β-specific coactivator-mimetic peptides using recombinant peptide libraries. Mol Endocrinol 14:605–622
34. Puigserver P, Adelmant G, Wu Z, Fan M, Xu J, O'Malley BW, Spiegelman BM 1999 Activation of PPARγ coactivator-1 through transcription factor docking. Science 286:1368–1371
35. Hong H, Yang L, Stallcup MR 1999 Hormone-independent transcriptional activation and coactivator binding by novel orphan nuclear receptor ERR3. J Biol Chem 274: 22618–22626
36. Xie W, Hong H, Yang NN, Lin RJ, Simon CM, Stallcup MR, Evans RM 1999 Constitutive activation of transcription and binding of coactivator by estrogen-related receptors 1 and 2. Mol Endocrinol 13:2151–2162
37. Wiebel FF, Steffensen KR, Treuter E, Feltkamp D, Gustafsson J-A 1999 Ligand-independent coregulator recruitment by the triply activatable OR1/retinoid X receptor-α nuclear receptor heterodimer. Mol Endocrinol 13:1105–1118
38. Tremblay A, Tremblay GB, Labrie F, Giguere V 1999 Ligand-independent recruitment of SRC-1 to estrogen receptor β through phosphorylation of activation function AF-1. Mol Cell 3:513–519
39. Kalkhoven E, Valentine JE, Heery DM, Parker MG 1998 Isoforms of steroid receptor co-activator 1 differ in their ability to potentiate transcription by the oestrogen receptor. EMBO J 17:232–243
40. McInerney EM, Rose DW, Flynn SE, Westin S, Mullen TM, Krones A, Inostroza J, Torchia J, Nolte RT, Assa-Munt M, Milburn MV, Glass CK, Rosenfeld MG 1998 Determinants of coactivator LXXLL motif specificity in nuclear receptor transcriptional activation. Genes Dev 12:3357–3368
41. Eng FCS, Barsalou A, Akutsu N, Mercier I, Zechel C, Mader S, White JH 1998 Different classes of coactivators recognize distinct but overlapping binding sites on the estrogen receptor ligand binding domain. J Biol Chem 273:28371–28377
42. Tzukerman MT, Esty A, Santiso-Mere D, Danielian P, Parker MG, Stein RB, Pike JW, McDonnell DP 1994 Human estrogen receptor transactivational capacity is determined by both cellular and promoter context and mediated by two functionally distinct intramolecular regions. Mol Endocrinol 8:21–30
43. Norris JD, Fan D, Stallcup MR, McDonnell DP 1998 Enhancement of estrogen receptor transcriptional activity by the coactivator GRIP-1 highlights that role of activation function 2 in determining estrogen receptor pharmacology. J Biol Chem 273:6679–6688
44. Norris JD, Fan D, Aleman C, Marks JR, Futreal PA, Wiseman RW, Iglehart JD, Deininger PL, McDonnell DP 1995 Identification of a new subclass of Alu DNA repeats which can function as estrogen receptor-dependent transcriptional enhancers. J Biol Chem 270: 22777–22782
45. Norris JD, Fan D, Wagner BL, McDonnell DP 1996 Identification of the sequences within the human C3 promoter required for estrogen responsiveness provides insight into the mechanism of tamoxifen mixed agonist activity. Mol Endocrinol 10:1605–1616